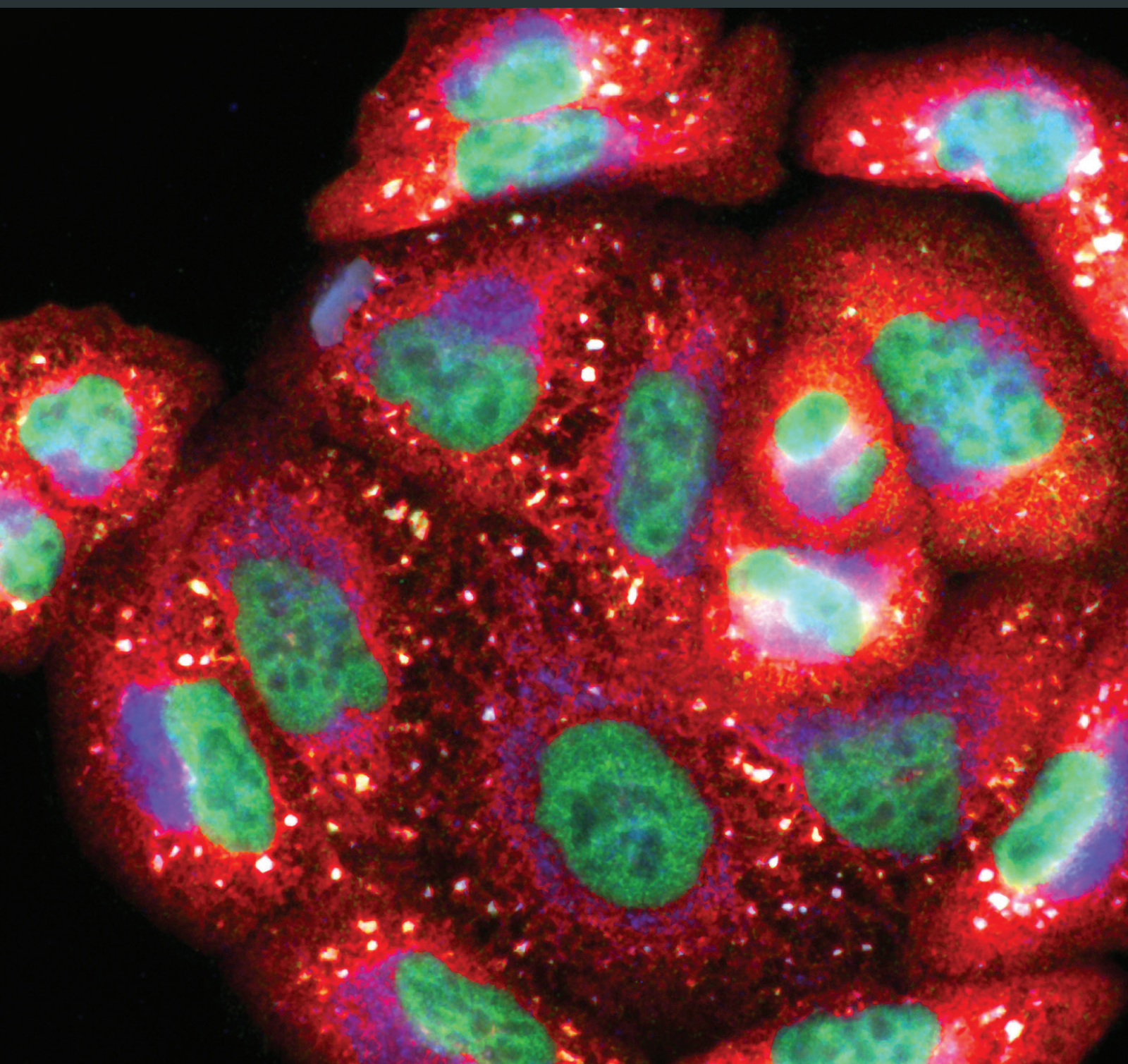
Oxidative Medicine and Cellular Longevity

Redox Imbalance and Stroke

Guest Editors: Ruidong Ye, Ming Shi, Qian Liu, and Jieli Chen



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Editorial Board

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Editorial
Redox Imbalance and Stroke

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Redox status is correlated with mitochondrial function, lipid raft turnover, and cellular cross talk in the neurovascular coupling. Redox imbalance is a hallmark event in the pathophysiology and prognosis of stroke. Excessive reactive oxygen species (ROS) can cause oxidative damage to various biological macromolecules including DNA, lipids, and proteins, thereby altering several signaling pathways that ultimately promote cellular damage and death. It is critical to address the relationship between redox status and stroke and to translate the accumulated mechanistic knowledge from animal models to clinical settings.

This special issue is devoted to highlighting novel findings on the role of redox imbalance in stroke and possibilities of the antioxidant supplementation for stroke treatment.

In this issue there are two investigations focused on the contribution of systemic inflammatory status to pathophysiology and prognosis of ischemic stroke patients. In a cross-sectional study, lower serum level of caveolin-1, which has been reported to maintain blood-brain barrier integrity and counteract oxidative stress, was found to be significantly related to cerebral microbleeds in patients with acute ischemic stroke. However, no relationship was observed between caveolin-1 and the presence of silent lacunar infarcts and white matter hyperintensities, two other types of cerebral small vessel disease. Though the relatively small sample size (~156 patients) may cripple the power of the evaluation, this study strongly supports caveolin-1 as a biomarker for predicting cerebral microbleeds in acute ischemic stroke. Maintaining caveolin-1 level appeared to be a potential strategy in treating cerebral small vessel diseases.

X. Zhang et al. carried out a prospective study on the predictive effects of the presence of metabolic syndrome (MetS) on early neurological deterioration following acute ischemic stroke. Interestingly, systemic markers of inflammation, fibrinogen and high-sensitivity C-reactive protein, were not the mediators of the relation between MetS and early neurological deterioration. The authors addressed that local oxidative or inflammatory response markers may be more appropriate when involved in studies of stroke and other potential correlated factors.

Two investigations focused on how comorbidity affects stroke outcome and therapeutic approaches. Stroke patients with diabetes mellitus have worsened mortality and neurological recovery. Bradykinin receptors have been demonstrated to play important roles in cerebral ischemia/reperfusion injury in the context of diabetes. R. Shi et al. carried out a study to elucidate the differential roles of bradykinin B1 receptor and B2 receptor underlying the effect of tissue kallikrein on the stroke outcome in diabetic rats. The results revealed that tissue kallikrein protects against inflammatory reactions by suppression of microglial activation and neutrophil infiltration through bradykinin B2 receptor, rather than B1 receptor. The bradykinin B2 receptor-dependent protection requires the activation of ERK/CREB/Bcl-2 signaling pathway to counteract apoptosis and neuroinflammation. Another potent antioxidant agent, minocycline, was investigated by E. A. Fontes-Júnior et al.

in cortical ischemic injury combined with chronic alcohol intoxication. The results showed that chronic alcohol intoxication increased nitrite and lipid peroxidation levels and neuronal loss caused by ischemia. Minocycline was effective in preventing histological and neurological damage caused by stroke alone. Nevertheless, the protection of minocycline was abolished when stroke was preceded by chronic alcohol intoxication. This study suggests that ischemic stroke combined with chronic alcohol intoxication is more severe and may require different therapeutic strategies.

J. Qu et al. provided a comprehensive review to summarize the role of ROS in the intracerebral hemorrhage. ROS is mainly released from neurons exposed to hemoglobin metabolic products after intracerebral hemorrhage, which is strikingly different from the pathophysiology in ischemic stroke. This ROS is harmful to the central nervous system through cell death and structural damage, especially the disruption of the blood-brain barrier. The review continues to discuss how endogenous antioxidant system interacts with the oxidant agents and why previous antioxidant therapy failed to confer practical neuroprotection in clinical trials. The authors suggest more attention be paid to the local and systemic influences of ROS in intracerebral hemorrhage and the longitudinal profile of ROS burst after intracerebral hemorrhage.

In another review article, J. Bu et al. discussed the effects of a natural product, omega-3 polyunsaturated fatty acids (n-3 PUFAs), the major component of fish oil, on the redox imbalance after stroke. Due to their antioxidation, anti-inflammation, and interaction with pathways such as Nrf2/HO-1 signaling, n-3 PUFAs are neuroprotective in reducing infarction volume and improving neurogenesis and revascularization. The authors also expressed that further studies were warranted to establish the specific treatment strategy of n-3 PUFAs in stroke.

In conclusion, the goal of this special issue is to drive a better understanding of how redox imbalance modulates pathophysiology and prognosis of stroke and how this can be harnessed as a therapeutic target in the stroke treatment. The original and review articles in this issue contributed by the experts in the fields of redox and stroke will nurture and expand knowledge of the mechanisms behind oxidative damage.

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Research Article

Tissue Kallikrein Alleviates Cerebral Ischemia-Reperfusion Injury by Activating the B2R-ERK1/2-CREB-Bcl-2 Signaling Pathway in Diabetic Rats

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Diabetes mellitus (DM) substantially increases the risk of ischemic stroke and reduces the tolerance to ischemic insults. Tissue kallikrein (TK) has been demonstrated to protect neurons from ischemia/reperfusion (I/R) injury in orthoglycemic model by activating the bradykinin B2 receptor (B2R). Considering the differential effects of B2R or bradykinin B1 receptor (B1R) on cardioprotection and neuroprotection in I/R with or without diabetes, this study was designed to investigate the role of TK during cerebral I/R injury in streptozotocin-induced diabetic rats. Intravenous injection of TK inhibited apoptosis in neurons, alleviated edema and inflammatory reactions after focal cerebral I/R, significantly reduced the infarct volume, and improved functional recovery. These beneficial effects were accompanied by activation of the extracellular signal-regulated kinase 1/2 (ERK1/2), cAMP response element-binding (CREB), and Bcl-2 signal proteins. Inhibition of the B2R or ERK1/2 pathway abated the effects of TK, whereas an antagonist of B1R enhanced the effects. These findings reveal that the neuroprotective effect of TK against cerebral I/R injury in streptozotocin-induced diabetic rats mainly involves the enhancement of B2R and ERK1/2-CREB-Bcl-2 signaling pathway activity.

1. Introduction

Ischemic stroke is the third leading cause of disability-adjusted life years worldwide [1]. Diabetes mellitus (DM) is one definitive risk factor of stroke and has been diagnosed in more than 30% of ischemic stroke patients [2]. Indeed, stroke survivors with diabetes have a higher risk of recurrence and a poorer prognosis compared with nondiabetics [3–6]. Although intensive glycemic control decreases the infarct volume and improves functional recovery after stroke [7], early recanalization is the only evidence-based effective therapy for improving the clinical outcomes of acute ischemic stroke [8]. Ischemia/reperfusion (I/R) injury, as one serious complication of restoring blood flow to the ischemic cerebrum, may offset the benefits of recanalization, especially in diabetes cases [9]. DM is known to exacerbate ischemic injury and to impede functional recovery by elevating inflammatory cytokines, promoting leukocyte infiltration, and accelerating the subsequent apoptotic cascade and neuronal death [5, 10–12]. Moreover, the cerebrum of diabetics is resistant to the neuroprotective effects of ischemic pre- or postconditioning or the administration of neuropharmacological agents [13]. Thus, further evaluation of the effects of DM is necessary to develop novel protective therapies for cerebral ischemia.

Kinins derived from kininogen produced by tissue kallikrein (TK) exert a broad spectrum of cellular functions via activation of the bradykinin B1 or B2 receptor (B1R or B2R, resp.) [14]. The physiological and pathological effects of TK have been shown to involve several cellular signaling pathways. Our previous studies have shown that TK could alleviate glutamate-induced neurotoxicity and protect cortical neurons against I/R and hypoxia/reoxygenation injury via the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway [15–17]. In nondiabetic animals, TK was shown to improve neurofunctioning after ischemic stroke by inhibiting the NF-κB signal pathway, activating the ERK1/2 pathway [18, 19], while in diabetic rats, TK attenuates insulin resistance and diabetic nephropathy via activation of phosphatidylinositol 3-kinase/protein kinase B [20]. Furthermore, increased TK plasma concentrations might exert greater cardioprotection in type 2 DM patients than that in non-DM patients [21]. Recently, we found a detrimental role of B1R and a beneficial effect of B2R in diabetic cerebral ischemia [22]. The present study aimed to assess whether TK could ameliorate cerebral I/R injury in streptozotocin-induced diabetic rats and investigate the roles of the ERK1/2 signaling pathway during acute ischemia and early reperfusion.

2. Materials and Methods

2.1. Establishment of the Diabetes Model. Male Sprague-Dawley rats (100–110 g) were purchased from the Animal Center of Jinling Hospital. All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications Number 80–23, revised 1996) and under the approval of the Institutional Animal Care and Use Committee of Nanjing University. All animals were placed on a 12/12-hour light/dark schedule and housed in a temperature- (22 ± 2°C) and humidity-controlled (55 ± 5%) room with free access to food (high-fat diet) and water. Diabetes was induced with streptozotocin (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 35 mg/kg after three weeks of high-fat diet feeding, as previously described [23]. Two weeks after the injection of streptozotocin, rats with fasting blood glucose concentrations ≥ 16.67 mmol/L (300 mg/dL) in three separate measures were considered diabetic.

2.2. Focal Cerebral Ischemia. Middle cerebral artery occlusion (MCAO) and reperfusion models in rats were established as previously described [24]. After 90 min of MCAO, the filament was withdrawn to restore cerebral blood flow (CBF) through the left MCA. CBF was monitored continuously with a PeriFlux Laser Doppler System 5000 (Perimed AB, Sweden) throughout the operation to confirm proper occlusion and reperfusion. After full recovery from anesthesia, rats were tested with the Longa score. The scores of 1 (failure to extend left forepaw fully), 2 (circling to the left), and 3 (falling to the left) represent mild focal neurologic deficit, moderate focal neurologic deficit, and severe focal deficit, respectively. The animal with a score of 4 could not walk spontaneously and experienced coma and confusion. The rats that attained a score of 0 were excluded from the study, which indicated noneurologic deficit. Sham-operated rats underwent the same operation without the insertion of the filament.

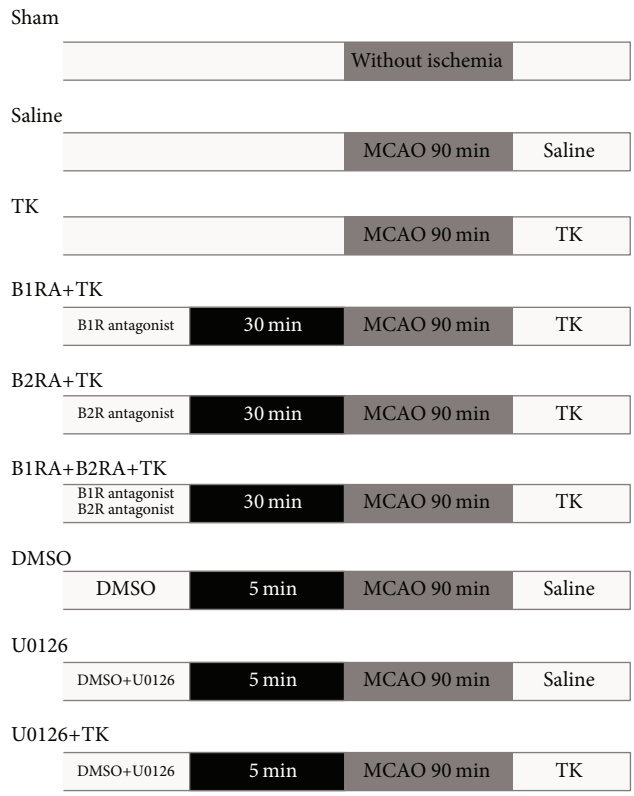

Figure 1: The experimental grouping and protocol.

2.3. Experimental Groups. Animals were randomized into nine groups: sham operation, saline, TK, TK+B1RA (B1R antagonist), TK+B2RA (B2R antagonist), TK+B1RA+B2RA, DMSO, U0126 (ERK1/2 inhibitor), and TK+U0126. Either the B2R antagonist bradyzide (1 nmol/kg, Sigma-Aldrich) or B1R antagonist Lys-(des-Arg9-Leu8)-bradykinin (300 nmol/kg, Sigma-Aldrich) was injected 30 min before the operation, whereas TK (1.6×10^{-2} PNAU/kg, Techpool Bio-Pharma Co. Ltd., Canton, China) [25] or saline (2 mL/kg) was given intravenously immediately after reperfusion. U0126 (9903, Cell Signaling Technology, Danvers, USA), an inhibitor of the ERK1/2 signaling pathway, was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) at 10 mmol/L and was subsequently diluted with saline for use. U0126 (400 μg/kg) or an equal amount of DMSO was administered intravenously 5 min before the operation [26]. The experimental grouping and protocol are described in the flow diagram (Figure 1). Previous studies demonstrated that the expression of B1R and B2R in cerebral ischemia/reperfusion injury peaks at 24 h after reperfusion in diabetes [22]. Therefore, 24 h after reperfusion was chosen as the time point for research in this study.

2.4. Assessment of Neurological Deficits. Neurological deficits were evaluated by an investigator blinded to the groupings at 24 h after reperfusion using the neurological severity scores (NSS) [27], which included motor test, sensory test, beam balance test, and examinations of reflexes and abnormal movements.

2.5. Estimation of Brain Edema. Rats were sacrificed 24 h after reperfusion. The hemispheres were weighed to obtain the wet weight (WW), and their dry weights (DW) were measured after desiccation at 105°C for 24 h. The brain moisture content (%) was calculated as previously described [22].

2.6. Evaluation of Infarct Volume. Brains were removed 24 h after reperfusion and cut into six 2 mm thick coronal slices. The infarct volume was evaluated by 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich, St. Louis, MO, USA) staining and analyzed according to our previous protocol [22].

2.7. Histopathological Study. For histopathological study, rats were perfused with 200 mL of 0.9% saline and 200 mL 4% paraformaldehyde (PFA, pH 7.4) successively through the left ventricle 24 h after reperfusion. Then, the cerebral hemisphere was collected and fixed with 4% PFA. After gradient elution with sucrose, the brain was quickly frozen and cut into 14 μm sections.

2.7.1. Fluoro-Jade C Staining (FJC). Slides were immersed in a basic alcohol solution consisting of 1% NaOH in 80% ethanol for 5 min and were then rinsed in 70% ethanol for 2 min, followed by incubation in 0.06% potassium permanganate solution for 10 min. The sections were then immersed in 0.0001% working solution of FJC (dissolved in 0.1% acetic acid vehicle) for 15 min. After washing and drying, the sections were cleaned in xylene and cover-slipped with DPX (Sigma-Aldrich, St. Louis, MO, USA) nonfluorescent mounting media. The data are expressed as the number of FJC-positive cells counted per section relative to the sham group.

2.7.2. TUNEL Staining. The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (Roche, Indianapolis, IN, USA) was used to assess apoptosis in neurons. Slides were postfixed with 4% PFA for 20 min and permeabilized with 0.1% Triton X-100 for 2 min on ice. After incubating in 15% glacial acetic acid for 2 min, the sections were treated with TUNEL mixture (enzyme solution : label solution = 1 : 9) for 60 min at 37°C in a dark humidified atmosphere, followed by converter-AP for 30 min. After washing with PBS three times for 5 min each, the specimens were incubated with BICP/NBT (Alkaline Phosphatase Color Development Kit, Beyotime, Shanghai, China) for 30 min and mounted under glass coverslips with CC/MOUNT. The data were expressed and analyzed in the same manner as for FJC staining.

2.7.3. Immunohistochemical Examination. Slides were postfixed in 4% PFA for 15 min followed by 0.3% H_2O_2 for 30 min. After permeabilization with 0.1% Triton for 10 min, the sections were incubated with 5% albumin bovine (Generay biotech, Shanghai, China) for 1 h. Then, the sections were incubated overnight with the following primary antibodies: anti-ionized calcium-binding adapter molecule 1 (Iba1) (1 : 1,000, Wako, 019-19741, Osaka, Japan), anti-cleaved caspase-3 (1 : 100, Cell Signaling Technology, Danvers, USA), or anti-myeloperoxidase (MPO) (1 : 200, Abcam, 65871 Cambridge, England). The slides were washed and then incubated with biotinylated anti-rabbit IgG antibody (Jackson, 711065152, West Grove, PA, USA) (1 : 200) for 2 h at room temperature. After incubation with ABC solution (Vector labs, California, USA), the slides were stained with the DAB Peroxidase Substrate Kit (Vector labs, California, USA), followed by PBS washing once the desired color was achieved. Sections were cleaned in xylene and cover-slipped with neutral balsam. The data were expressed and analyzed in the same manner as for FJC staining.

2.8. Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction. Total RNA was extracted from ischemic boundary region tissue at 24 h after reperfusion using the Trizol reagent (Sigma-Aldrich, St. Louis, MO, USA) and then reverse-transcribed into cDNA using a RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Waltham, MA, USA) following the manufacturer's recommendations. Quantitative real-time PCR was performed using a real-time PCR system (Agilent Technologies, Santa Clara, CA, USA) with a fluorescent dye (CW0956 UltraSYBR Mixture). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous reference gene. The normalized messenger RNA levels were previously described in detail [28]. Data are expressed as the ratio of the level of IL-1β or TNF-α to that of GAPDH in the treated groups relative to the sham group. The primer sequences for the genes were as follows: IL-1β: sense: 5′-AGACTTCACAGAGGATACCAC-CCAC-3′, antisense: 5′-CAATCAGAATTGCCATTGCAC-AA-3′; TNF-α: sense: 5′-AGCAAACCACCAAGCGGAGG-3′, antisense: 5′-CAGCCTTGTCCCTTGAAGAGAAC-3′; and GAPDH: sense: 5′-GCAAGTTCAACGGCACAG-3′, antisense: 5′-GCCAGTAGACTCCACGACAT-3′.

2.9. Western Blotting. Samples for western blotting were collected from brain infarction boundary regions. After extraction and quantification, equal amounts of protein were separated by sodium dodecyl sulfate-PAGE and then transferred to an immobilon-polyvinylidene fluoride membrane. The membranes were blocked using 5% milk for 2 h at room temperature and probed with the primary antibody overnight at 4°C. These primary antibodies included rabbit anti-phospho-ERK1/2 (1 : 5,000), rabbit anti-total-ERK1/2 (1 : 5,000), rabbit anti-phospho-cAMP response element-binding protein (CREB) (1 : 1,000), rabbit anti-total-CREB (1 : 1,000), rabbit anti-Bcl-2 (1 : 200), rabbit anti-Bax (1 : 400), rabbit anti-cleaved caspase-3 (1 : 1000), and rabbit anti-β-actin (1 : 5,000). The secondary antibody was anti-rabbit

HRP-conjugated antibody (1 : 4,000). All primary antibodies were purchased from Cell Signaling Technology (Danvers, USA), with the exception of the Bcl-2 antibody purchased from Santa Cruz (Dallas, USA). Immunoblots were developed with the Immobilon ECL method (Millipore, Billerica, Massachusetts, USA). Gray bands were converted to density values using Image J software for quantification analysis.

2.10. Data Analysis and Statistics. All data except for the NSS values are expressed as mean ± SD. The neurologic tests scores were analyzed using the Kruskal-Wallis test followed by the Mann-Whitney U test with Bonferroni corrections. Differences between groups were compared using one-way analysis of variance with the Welch correction for unequal variances. Post hoc tests, such as Bonferroni or Dunnett's T3 multiple comparison tests, were used according to whether unequal variances were present. A two-sided P value of $P < 0.05$ was defined as significant.

3. Results

3.1. Modifications of Neurological Deficits. At 24 h after MCAO, neurologic function improved in the TK group compared with the saline group ($P < 0.05$, Figure 2(a)). Compared with the TK group, the B1RA+B2RA+TK and B2RA+TK groups showed higher neurological deficit scores ($P < 0.05$), whereas the B1RA+TK group showed a comparable score ($P > 0.05$). Furthermore, neurological function in the B1RA+B2RA+TK group was worse than that of the B1RA+TK group ($P < 0.05$) but similar to that of the B2RA+TK group ($P > 0.05$). Treatment with the ERK1/2 inhibitor U0126 significantly aggravated neurological functioning ($P < 0.05$) and compromised the beneficial effect of TK (TK versus U0126+TK, $P < 0.05$).

3.2. Variation of Brain Edema. Brain edema at 24 h after reperfusion was significantly alleviated in TK group compared to saline group ($P < 0.05$, Figure 2(b)). B1R antagonist pretreatment had no interference on TK. However, pretreatment with the B2R antagonist hindered the efficacy of TK ($P < 0.05$). Brain edema in the B1RA+B2RA+TK group was more severe than that in the TK and B1RA+TK groups but less than that in the B2RA+TK group ($P < 0.05$). Administration of U0126 alone clearly increased the brain water content compared to rats in the saline group ($P < 0.05$). However, the brain water content in the U0126+TK group was not different from that in the TK group.

3.3. Change in Cerebral Infarct Volume. TK treatment remarkably reduced the infarct volume compared to saline at 24 h after reperfusion ($P < 0.05$, Figures 2(c) and 2(d)). Administration of B1RA+TK could further reduce the infarct volume of TK-treated animals, whereas B2RA+TK or B1RA+B2RA+TK treatment abolished the beneficial effect observed with the TK intervention ($P < 0.05$). Additionally, the infarct volume in the B1RA+B2RA+TK group was lower than that in the B2RA+TK group but higher than that in the B1RA+TK group ($P < 0.05$). Treatment with the ERK1/2 inhibitor U0126 increased the infarct volume in the saline group ($P < 0.05$). Furthermore, the infarct volume with U0126+TK treatment was higher than that with TK treatment alone ($P < 0.05$).

3.4. Expression of Iba1, MPO, and Proinflammatory Cytokines. Iba1 is a protein biomarker expressed in microglia that is upregulated during inflammatory responses after stroke in the infarction border zone. MCAO followed by 24 h of reperfusion induced a 20.46 ± 2.40-fold increase in Iba1-positive cells. Compared to saline, TK intervention significantly suppressed the activation of Iba1 ($P < 0.05$, Figures 3(A) and 3(B)). Furthermore, the efficacy of TK was enhanced by pretreatment with the B1R antagonist but was abolished by the B2R antagonist ($P < 0.05$). The number of Iba1-positive cells in the B1RA+B2RA+TK group was higher than that in the TK and B1RA+TK groups but lower than that in the B2RA+TK group ($P < 0.05$). In addition, U0126 treatment led to an increase in microglial activation (U0126 versus saline, $P < 0.05$), while pretreatment with U0126 abated the effect of TK (U0126+TK versus TK, $P < 0.05$). Neutrophil infiltration into the ischemic regions was investigated by immunohistochemical staining for MPO (Figures 3(C) and 3(D)), and the change in MPO expression was similar to that observed for Iba1.

Proinflammatory cytokines are expressed in the ischemic core in the early stage of the brain ischemic model. Reperfusion for 24 h after MCAO (saline group) led to a 61.27 ± 7.46-fold increase in IL-1β and a 30.71 ± 9.64-fold increase in TNF-α compared with the sham group (Figures 4(a) and 4(b)). Treatment with TK ameliorated this upregulation compared with the saline group ($P < 0.05$). Compared to TK-treated animals, B1R antagonism prior to MCAO attenuated IL-1β expression, whereas B2R antagonism elevated the levels of IL-1β and TNF-α ($P < 0.05$). Simultaneous antagonism of B1R and B2R before TK treatment induced much higher levels of IL-1β and TNF-α than TK intervention alone ($P < 0.05$). Furthermore, the relative expression of IL-1β and TNF-α in the B1RA+TK group was lower than that in the B1RA+B2RA+TK group ($P < 0.05$), whereas the level of IL-1β in the B1RA+B2RA+TK group was lower than that in the B2RA+TK group ($P < 0.05$). After injection of the ERK1/2 inhibitor U0126, the relative expression of TNF-α and IL-1β was dramatically increased compared to the saline group ($P < 0.05$). Additionally, pretreatment with U0126 elevated the level of TNF-α in rats that received TK treatment (TK versus U0126+TK, $P < 0.05$).

3.5. Neuronal Degeneration and Apoptosis. Neuronal degeneration was evaluated by FJC staining. Relative to the sham operation group, 24 h of reperfusion after MCAO stimulated a 19.12 ± 2.92-fold increase in FJC-positive cells in the ischemic cortex. Administration of TK decreased the relative density of FJC-positive cells compared to saline ($P < 0.05$, Figures 5(A) and 5(B)). The efficacy of TK treatment was enhanced with the B1R antagonist but reversed with the B2R antagonist ($P < 0.05$). The number of degenerated neurons in the B1RA+B2RA+TK group was significantly greater than

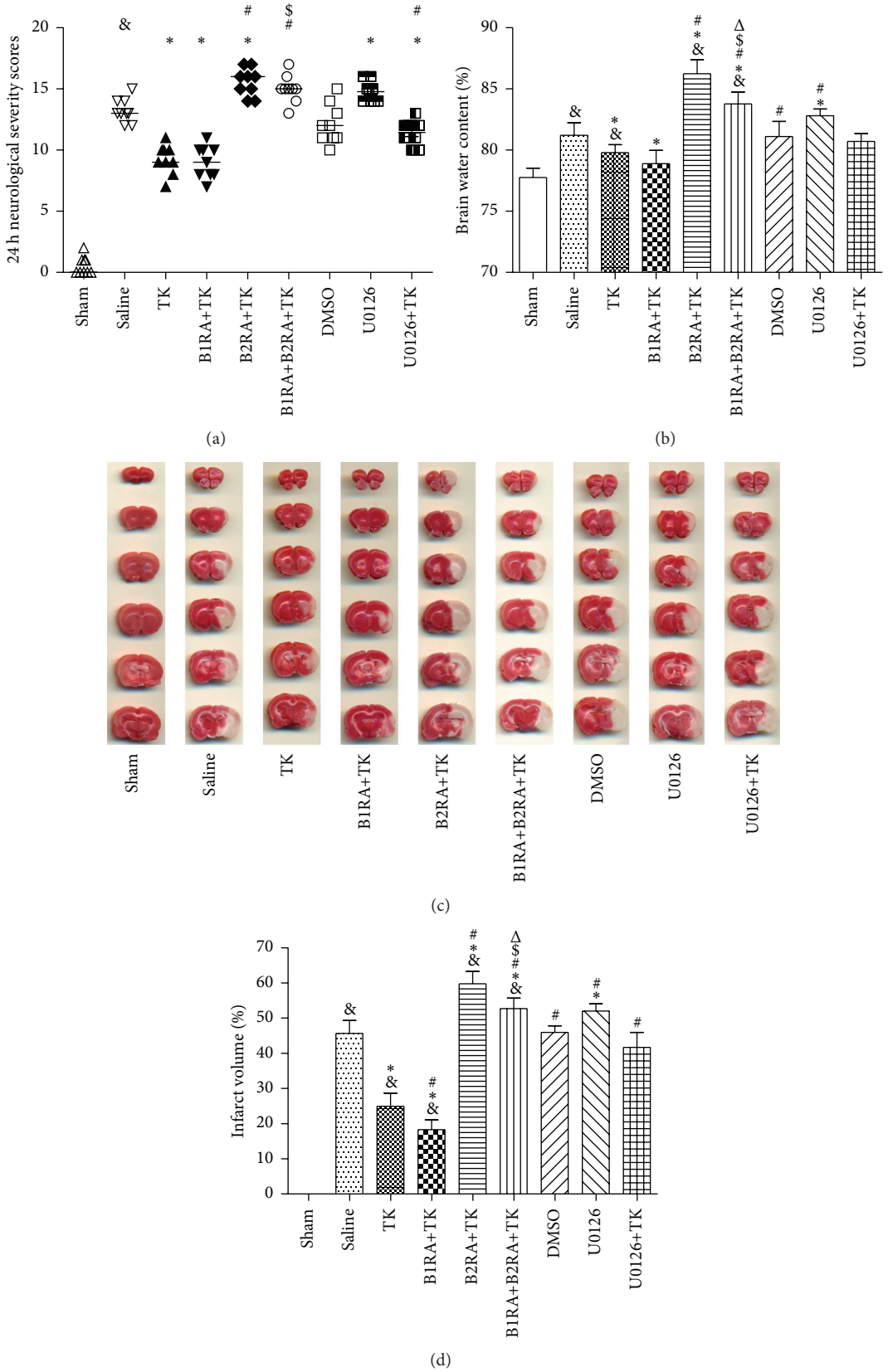

Figure 2: (a) NSS values recorded for each animal at 24 h after MCAO. $n = 9$ rats/group. (b) Brain edema recorded in each animal at 24 h after MCAO. Data are expressed as mean ± SD; $n = 9$ rats/group. (c) 2,3,5-Triphenyltetrazolium chloride staining recorded in nine groups at 24 h after MCAO; (d) the infarct volume percent at 24 h after MCAO. Data are expressed as mean ± SD; $n = 7$ rats/group ($^{\&}P < 0.05$ versus sham group; $^{*}P < 0.05$ versus saline group; $^{\#}P < 0.05$ versus TK group; $^{\$}P < 0.05$ versus B1RA+TK group; $^{\Delta}P < 0.05$ versus B2RA+TK group).

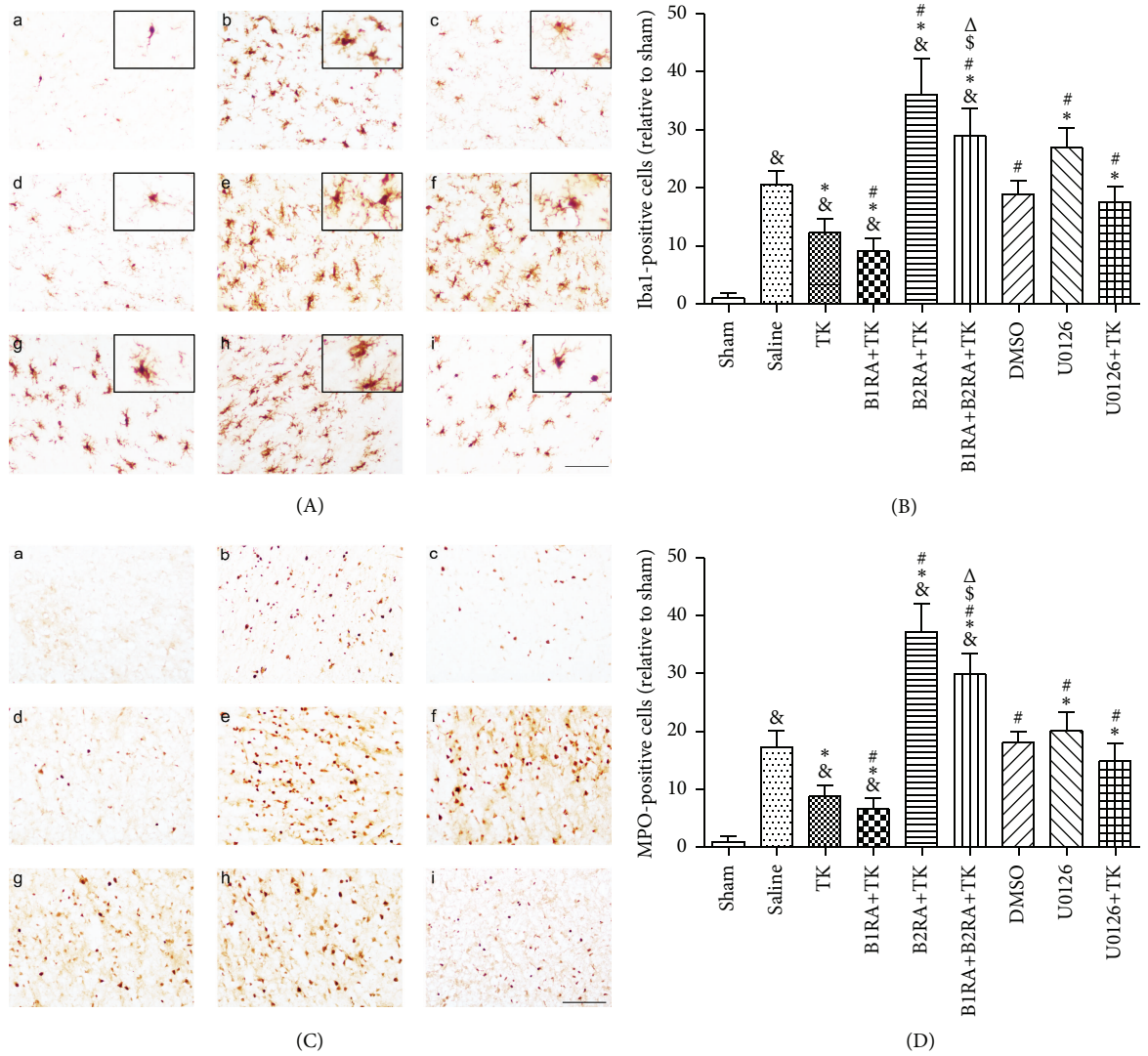

FIGURE 3: (A) Iba1 staining was performed 24 h after MCAO to observe microglia. (B) The number of Iba1-positive cells was counted at 24 h after MCAO. (C) MPO staining was performed 24 h after MCAO to observe neutrophils. (D) The number of MPO-positive cells was counted 24 h after MCAO. (a) Sham; (b) saline; (c) TK; (d) B1RA+TK; (e) B2RA+TK; (f) B1RA+B2RA+TK; (g) DMSO; (h) U0126; (i) U0126+TK. Scale bar = 100 mm. Data are expressed as mean ± SD; $n = 3$ rats/group. Six representative microscopic fields were analyzed for each rat ($^{\&}P < 0.05$ versus sham group; $^{*}P < 0.05$ versus saline group; $^{\#}P < 0.05$ versus TK group; $^{\$}P < 0.05$ versus B1RA+TK group; $^{\Delta}P < 0.05$ versus B2RA+TK group).

that in the TK and B1RA+TK groups but less than that in the B2RA+TK group ($P < 0.05$). U0126 pretreatment aggravated neuronal injury ($P < 0.05$) and weakened the benefit of TK ($P < 0.05$).

TUNEL staining and immunohistochemical staining for cleaved caspase-3 were used to verify the antiapoptosis effect of TK in the ischemic cortex. The presence of apoptotic cells in each group was consistent with the extent of degenerated neurons (Figures 5(C), 5(D), 5(E), and 5(F)).

3.6. Activity of the ERK1/2 Signaling Pathway. Compared to corresponding expressions before MCAO, the relative levels of p/T-ERK1/2 and p/T-CREB showed remarkable increases with reperfusion; these levels showed fold increases of 2.6 and 2.8 after 1 h of reperfusion, respectively ($P < 0.05$, Figure 6). Thus, 1 h of reperfusion was chosen as the optimal time point for measuring the expression of signaling proteins.

TK treatment enhanced the relative expression of p/T-ERK1/2, p/T-CREB, and Bcl-2/β-actin compared to the sham group at 1 h of reperfusion after MCAO, with higher values compared to the saline group ($P < 0.05$, Figure 7). Pretreatment with a B1R antagonist further elevated the levels of p/T-CREB and Bcl-2/β-actin in TK-treated animals ($P < 0.05$). However, the B2R antagonist abated the upregulation of p/T-ERK1/2, p/T-CREB, and Bcl-2/β-actin generated by TK treatment, and simultaneous pretreatment with the B1R and B2R antagonists had a similar effect ($P < 0.05$). However, the levels of all of the above proteins in the B1RA+TK group were higher than those in the B1RA+B2RA+TK group, whereas the

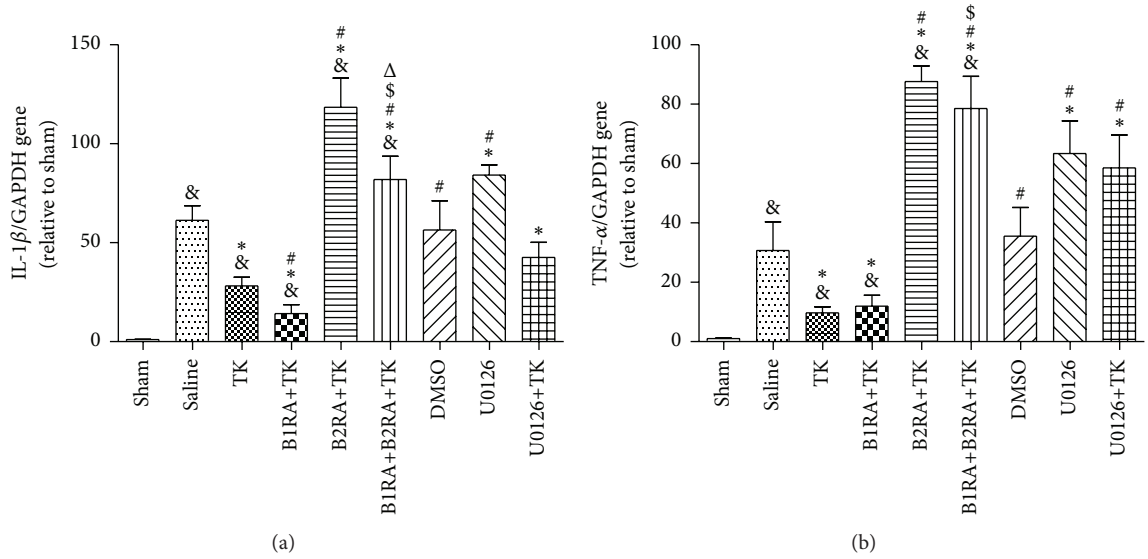

FIGURE 4: (a) The levels of IL-1β 24 h after MCAO. (b) The levels of TNF-α 24 h after MCAO. Values are expressed as mean ± SD; $n = 6$ rats/group ($^{\&}P < 0.05$ versus sham group; $^{*}P < 0.05$ versus saline group; $^{\#}P < 0.05$ versus TK group; $^{\$}P < 0.05$ versus B1RA+TK group; $^{\Delta}P < 0.05$ versus B2RA+TK group).

p/T-ERK1/2 and Bcl-2/β-actin levels in the B2RA+TK group were significantly lower ($P < 0.05$). In contrast, the elevated levels of Bax and cleaved caspase-3 expression stimulated by I/R were downregulated by TK treatment ($P < 0.05$). The inhibitory effect of TK on Bax expression was further amplified by the B1R antagonist but was weakened by the B2R antagonist ($P < 0.05$). The suppression effect of TK on cleaved caspase-3 was not changed significantly by B1R antagonist but impaired by B2R antagonist ($P < 0.05$). Furthermore, administration of B1RA+B2RA+TK decreased the levels of Bax and cleaved caspase-3 compared to those observed in the B2RA+TK group but elevated the levels in comparison to the B1RA+TK group ($P < 0.05$).

Compared to saline treatment, injection of U0126 before MCAO resulted in decreased expression of p/T-ERK1/2, p/T-CREB, and Bcl-2/β-actin compared to the saline group ($P < 0.05$, Figure 7). Additionally, U0126 treatment increased the levels of Bax and cleaved caspase-3 relative to the saline group ($P < 0.05$). Moreover, pretreatment with U0126 impaired the effects of TK treatment ($P < 0.05$). During the experiments, the total immunoreactive ERK1/2 and CREB levels were rarely changed by MCAO surgery or other interventions.

4. Discussion

In this study, we observed the neuroprotective effects of TK on I/R injury in streptozotocin-induced diabetic rats, involving the reduction of brain edema and infarct volume, suppression of inflammation and apoptosis, and amelioration of ischemia-induced behavioral deficits. TK led to upregulated expression of p-ERK1/2, p-CREB, and Bcl-2 proteins and downregulated expression of Bax and cleaved caspase-3 proteins in the cerebral I/R injury of diabetic rats. Additionally, the neuroprotective effects of TK were promoted by a B1R antagonist but were abrogated by treatment with a B2R antagonist or ERK1/2 inhibitor.

Early inflammation contributes to brain damage following I/R. Activated microglia and neutrophils are central to the inflammatory response and can release cytokines. Furthermore, neuronal degeneration and apoptosis and inflammatory responses in ischemic areas can be aggravated by hyperglycemia [29]. Previous studies have shown that hyperglycemia enhances microglia activation and neutrophil migration, which further worsened postischemia injury [30, 31]. This study revealed that immediate TK treatment after MCAO attenuated I/R-induced neuronal degeneration and cellular apoptosis, inhibited neutrophil migration and microglia activation, and reduced the levels of proinflammatory cytokines in diabetic rats. Furthermore, there were notably improved neurological outcomes in the TK-treated rats, indicating TK's involvement in the brain I/R process.

Pharmacological B2R blockade before TK treatment could boost neuron loss, microglia activation, neutrophil migration, and the levels of proinflammatory cytokines, suggesting that the neuroprotective effects of TK were mainly mediated by B2R. Our observations are consistent with the published literature showing that TK protects neurons from ischemic injury through activation of B2R [32]. Additionally, TK was demonstrated to inhibit I/R-induced apoptosis and inflammation in the brains of nondiabetic rats, and the efficacy was blocked by B2R antagonists [33]. However, in the present study, blockade of B1R before MCAO facilitated the actions of TK, indicating that activation of B1R exaggerated cerebral I/R injury in diabetic rats. Our recent findings showed that the upregulation of B1R in diabetic rats aggravated cerebral I/R injury compared with nondiabetic rats. Therefore, the B1R pharmacological antagonist plays a neuroprotective role in acute ischemia in diabetic rats [22],

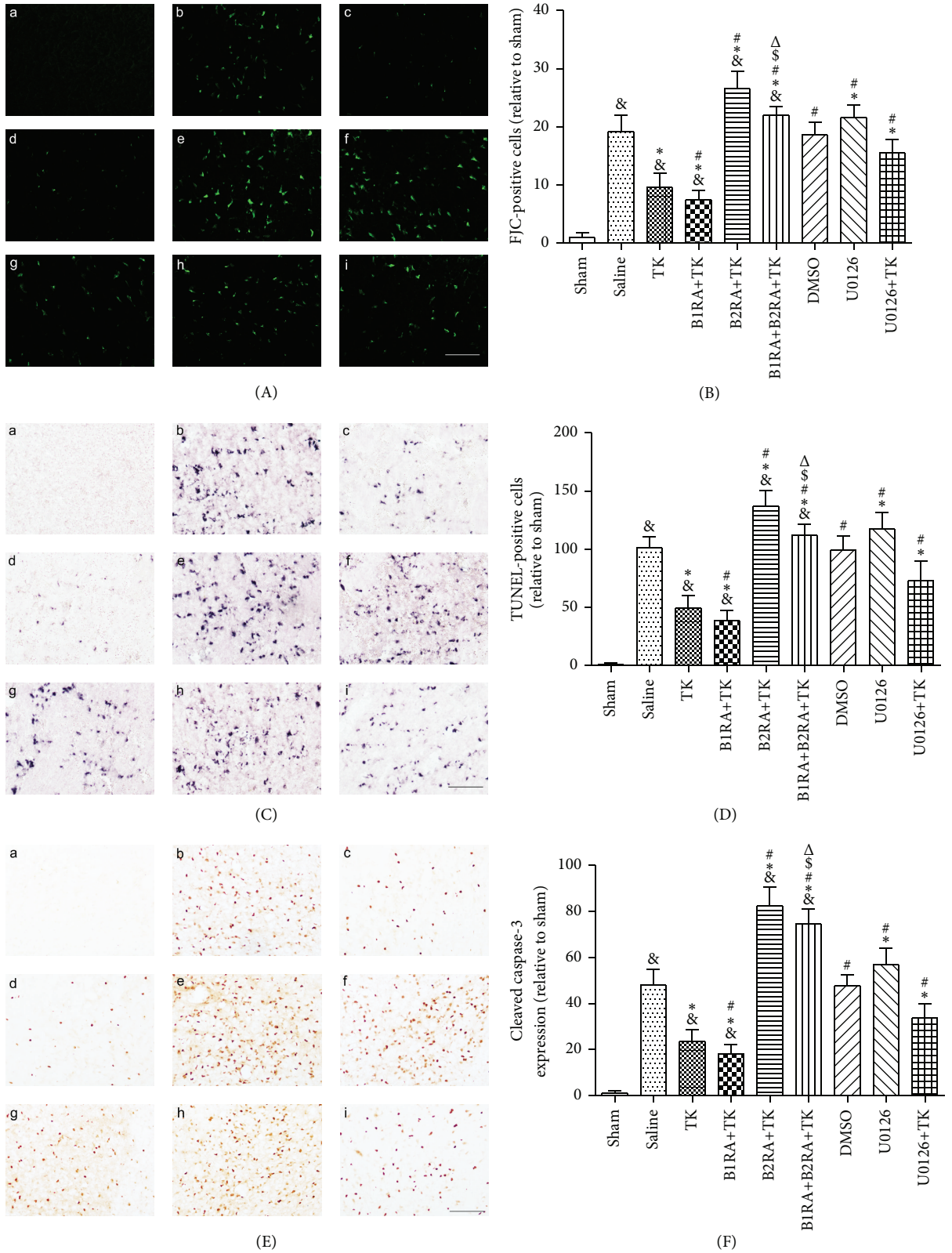

FIGURE 5: (A) FJC staining was performed 24 h after MCAO to assess neuron degeneration. (B) The number of FJC-positive cells was counted 24 h after MCAO. (C) TUNEL staining was performed 24 h after MCAO to assess apoptosis in neurons. (D) The number of TUNEL-positive cells was counted 24 h after MCAO. (E) Cleaved caspase-3 staining was performed 24 h after MCAO to assess neuron apoptosis. (F) The number of cleaved caspase-3-positive cells was counted after MCAO. (a) Sham; (b) saline; (c) TK; (d) B1RA+TK; (e) B2RA+TK; (f) B1RA+B2RA+TK; (g) DMSO; (h) U0126; (i) U0126+TK. Scale bar = 100 mm. Data are expressed as mean ± SD; $n = 3$ rats/group. Six representative microscopic fields were analyzed for each rat ($^{\&}P < 0.05$ versus sham group; $^{*}P < 0.05$ versus saline group; $^{\#}P < 0.05$ versus TK group; $^{\$}P < 0.05$ versus B1RA+TK group; $^{\Delta}P < 0.05$ versus B2RA+TK group).

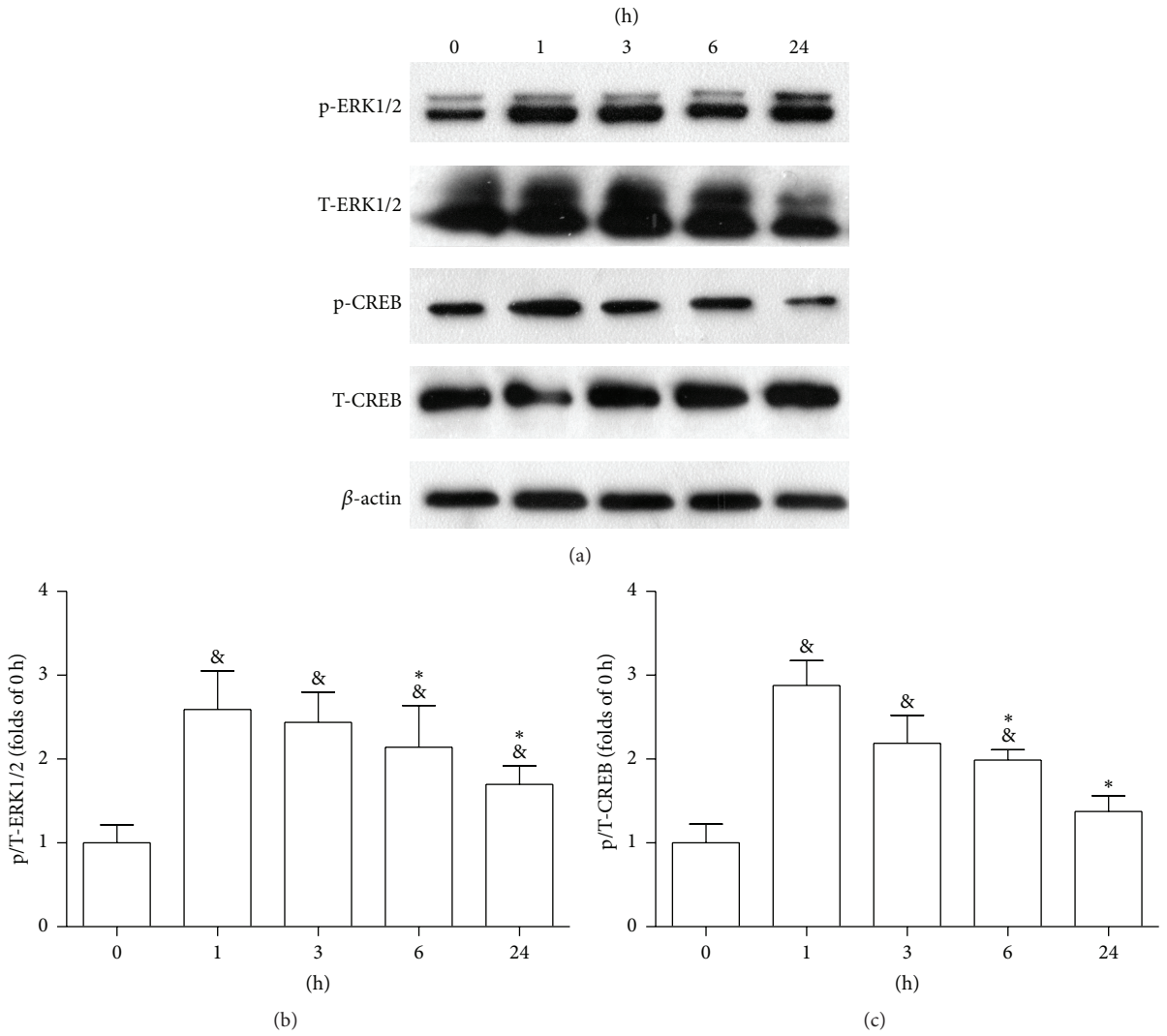

Figure 6: (a) Western blotting was performed to evaluate the expression levels of p-ERK1/2 and p-CREB. Brain tissues were extracted at 0 h, 1 h, 3 h, 6 h, and 24 h after MCAO. The phosphorylation of ERK1/2 (b) and CREB (c) peaked at 1 h. Data are expressed as mean ± SD; $n = 3$ rats/group ($^{\&}P < 0.05$ versus 0 h; $^{*}P < 0.05$ versus 1 h).

and this phenomenon is consistent with a report demonstrating that B1R-knockout nondiabetic mice suffering brain I/R injury have smaller infarct volumes and less postischemic inflammation [34]. Therefore, a combination of the B1R antagonist with TK treatment may provide a novel strategy for the treatment of stroke with diabetes. Additionally, simultaneous antagonism of B1R and B2R reversed the beneficial effects of TK, suggesting that the protection induced by TK was mediated primarily by B2R, although B1R activated by TK showed a detrimental effect on brain I/R injury in diabetic rats [22]. Thus, our results demonstrated that activation of B2R in cerebral I/R damage in diabetic rats suppressed inflammation, enhanced cell survival, and improved neurological functions, whereas activation of B1R produced the opposite effects.

A previous study on myocardial ischemia demonstrated a cardioprotective effect of the B2R agonist in nondiabetic mice and the B1R agonist in diabetes via inhibition of GSK-3β [35]. Possible explanations for this discrepancy include differences in animal species, tissues, organs, and I/R injury models. Moreover, in our findings, inactivation of B2R before MCAO resulted in a lower level of ERK1/2 phosphorylation than TK intervention alone in the ischemic brain, which was contrary to B1R inactivation. These observations suggest that activation of B2R signaling induced by TK leads to the phosphorylation of ERK1/2 in the diabetic cerebrum, indicating that B2R may be the main upstream protein in the ERK1/2 signaling pathway activated by TK.

The ERK1/2 pathway is known to be activated by various stimuli, such as oxidative stress, ionic imbalance, activation of glutamate receptors, and growth factors [36], and this pathway also plays a role in TK-mediated protection against I/R injury in orthoglycemic conditions [17–19]. Previous studies have demonstrated that the stimulation of B2R caused NO

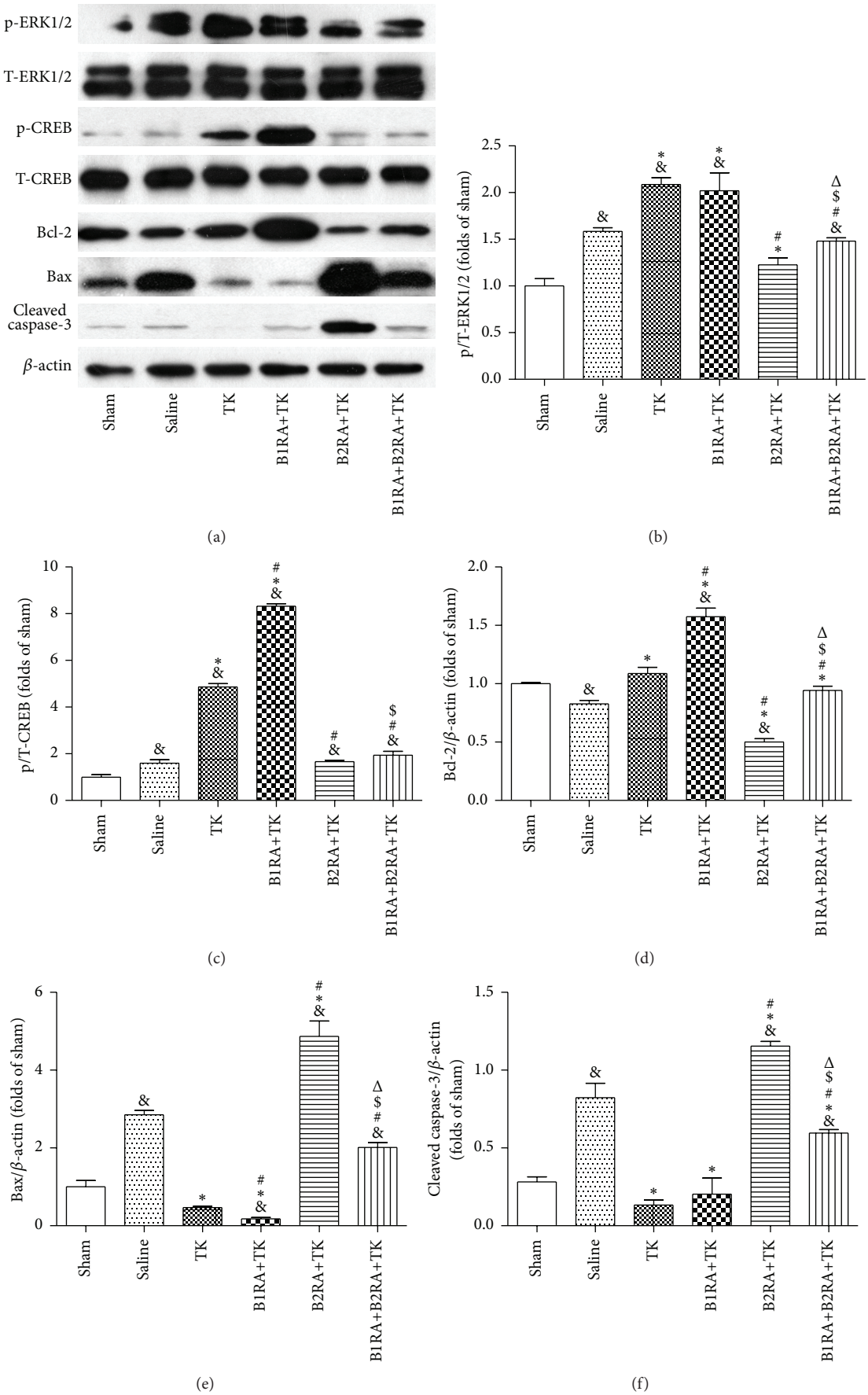

Figure 7: Continued.

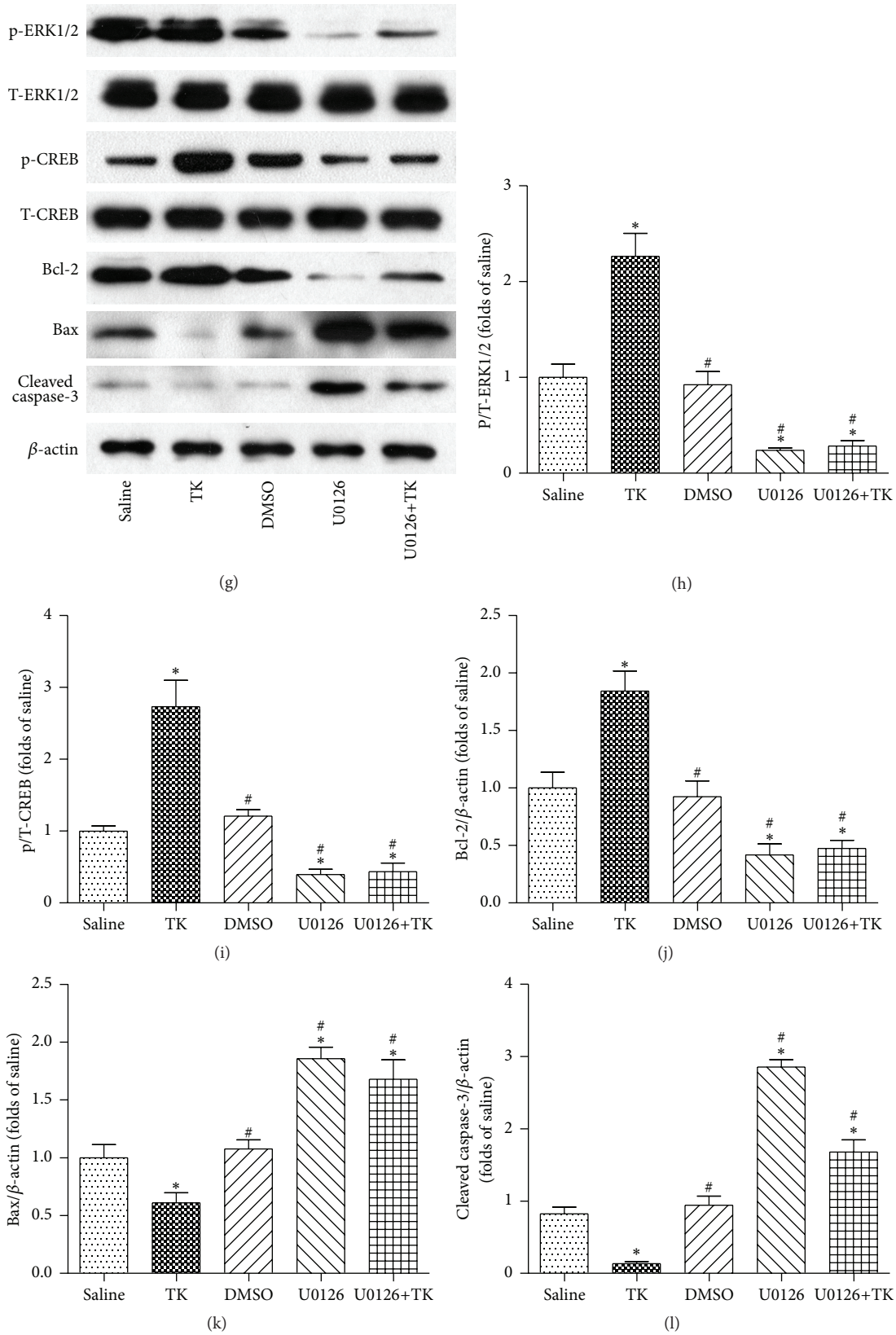

Figure 7: (a) Western blotting was performed to evaluate the activation levels of p-ERK1/2, p-CREB, Bcl-2, and Bax. The levels of p-ERK1/2 (b), p-CREB (c), Bcl-2 (d), Bax (e), and cleaved caspase-3 (f) are expressed as mean ± SD; $n = 4$. (g) Western blotting was performed to evaluate the activation levels of p-ERK1/2, p-CREB, Bcl-2, and Bax. The levels of p-ERK1/2 (h), p-CREB (i), Bcl-2 (j), Bax (k), and cleaved caspase-3 (l) are expressed as mean ± SD; $n = 3$ ($^{\&}P < 0.05$ versus sham group; $^{*}P < 0.05$ versus saline group; $^{\#}P < 0.05$ versus TK group; $^{\$}P < 0.05$ versus B1RA+TK group; $^{\Delta}P < 0.05$ versus B2RA+TK group).

production and NO could stimulate ERK [37, 38]. Therefore, the activation of B2R may lead to the upregulation of p-ERK1/2. Here, we also observed that the ERK1/2 pathway was one of the main signaling pathways involved in TK-mediated anti-inflammation and antiapoptosis following cerebral I/R in diabetes. TK treatment during reperfusion after MCAO in diabetic rats induced upregulation of p-ERK1/2, whereas pretreatment with the ERK1/2 pathway inhibitor U0126 before MCAO produced adverse effects that were similar to those for the B2R antagonist. U0126 was reported to significantly aggravate neurological dysfunction, increase infarct volumes and edema, and exacerbate inflammation and apoptosis [39, 40]. Our findings further showed that pretreatment with U0126 could partially but significantly counteract the efficacy of TK against cerebral I/R injury in diabetic rats. This phenomenon hints that the actions of TK against brain I/R injury in diabetic rats might involve other pathways besides the ERK1/2 signaling pathway. However, the ERK1/2 inhibitor completely suppressed the activation of p-ERK1/2, p-CREB, and Bcl-2 and promoted the activation of Bax and cleaved caspase-3 in TK-treated group, indicating that ERK1/2 may be the main upstream protein in this signaling pathway activated by TK.

CREB, as a transcription factor, participates in synaptic plasticity, memory. and survival. Phosphorylation of CREB by p-ERK1/2 not only displays neuroprotection in stroke animals [36, 41] but also prevents postischemic inflammation and neuronal damage [42]. Our data showed that the level of phosphorylated CREB changed with the expression of p-ERK1/2. Phosphorylated CREB was reported to stimulate expression of the antiapoptotic protein Bcl-2 and the reduction of caspase-3, which are associated with neuronal survival [43–46]. Bax and Bcl-2 are members of the Bcl-2 protein family and are associated with neuronal apoptosis. Our study revealed that TK could stimulate the signal pathway of ERK1/2-CREB-Bcl-2 and suppress the Bax and cleaved caspase-3 expressions. Additionally, pretreatment with the B1R antagonist strengthened the actions of TK on the abovementioned signaling pathways, whereas the B2R antagonist or ERK1/2 inhibitor U0126 produced the opposite effects on these signaling proteins. We also noticed sharp increases in degenerative and apoptotic neurons as well as inflammatory reactions after inhibition of the B2R or ERK1/2 pathway. The present findings were consistent with a previous study in which U0126 was shown to counteract the neuroprotection of ERK-CREB in transient global ischemia [47]. It is likely that the anti-inflammatory activity induced by TK through B2R involves enhanced phosphorylation of ERK1/2 and subsequently increases the phosphorylation of CREB, which in turn suppresses proinflammatory cytokine secretion in the damaged tissue and reduces microglia and neutrophil migration. According to our observations, TK might produce antiapoptotic effects through B2R and activation of the ERK1/2-CREB pathway, in addition to promoting Bcl-2 formation, suppressing Bax and cleaved caspase-3 expressions, and reducing cell degeneration and apoptosis.

Various drugs for stroke were neuroprotective preclinically but proved unsuccessful in clinical trials subsequently. Using healthy animals as the model to evaluate the efficacy of candidate drugs may be one of the most common problems. Recommendations issued by Stroke Therapy Academia Industry Roundtable (STAIR) Committee showed that drug intended for clinical trials should be administrated to animals accompanied with hypertension, diabetes, and hypercholesterolemia if it was demonstrated to be effective in healthy animals [46]. TK was demonstrated to offer neuroprotection by previous work in orthoglycemic models; however, the research on the role of TK in stroke animals with diabetes remains scarce. Therefore, it is meaningful to prove that TK could ameliorate the prognosis of I/R injury in hyperglycemic conditions.

In conclusion, early administration of TK could provide robust resistance to I/R damage in the diabetic brain. This neuroprotective effect was attributed, in the present study, to the antiapoptotic and anti-inflammatory effects mediated primarily through B2R and the ERK1/2-CREB-Bcl-2 pathway. Thus, our findings support the use of TK as a therapeutic approach to reduce the effects of brain I/R insults in diabetic subjects.

Competing Interests

All authors declare that there is no conflict of interests.

Authors' Contributions

Ruifeng Shi, Kunxiong Yuan, and Bin Hu contributed equally to this work.

Acknowledgments

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Review Article

The Role of Omega-3 Polyunsaturated Fatty Acids in Stroke

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Stroke is the third commonest cause of death following cardiovascular diseases and cancer. In particular, in recent years, the morbidity and mortality of stroke keep remarkable growing. However, stroke still captures people attention far less than cardiovascular diseases and cancer. Past studies have shown that oxidative stress and inflammation play crucial roles in the progress of cerebral injury induced by stroke. Evidence is accumulating that the dietary supplementation of fish oil exhibits beneficial effects on several diseases, such as cardiovascular diseases, metabolic diseases, and cancer. Omega-3 polyunsaturated fatty acids (n-3 PUFAs), the major component of fish oil, have been found against oxidative stress and inflammation in cardiovascular diseases. And the potential of n-3 PUFAs in stroke treatment is attracting more and more attention. In this review, we will review the effects of n-3 PUFAs on stroke and mainly focus on the antioxidant and anti-inflammatory effects of n-3 PUFAs.

1. Introduction

Stroke, also known as cerebrovascular insult or brain attack, is defined by World Health Organization as "neurological deficit of cerebrovascular cause that persists beyond 24 hours or is interrupted by death within 24 hours" in the 1970s [1]. Stroke was firstly reported in the 2nd millennium BC, and firstly described by Hippocrates. But up till now, the systematic treatment strategy of stroke remains elusive.

In 1946, Hansen and Burr found that the Eskimos who live in Newfoundland rarely suffer from cardiovascular disease [2]. They owe the beneficial effects to the diet of Eskimos, which is rich in fish and seafood. Fish oil begins to capture people's attention. Further studies indicated that the benefit effects of fish oil are mainly mediated by omega-3 polyunsaturated fatty acids (n-3 PUFAs), which are against a range of diseases, including cardiovascular diseases, inflammatory diseases like arthritis, metabolic diseases like type 2 diabetes, and cancer [3].

The aim of this paper is to summarize the research progress of n-3 PUFAs, especially the effects on stroke.

2. Subsets, Sources, and Metabolism of n-3 PUFAs

According to the number of double bonds in fatty acid side chains, the natural fats are classified into 3 subsets: saturated, monounsaturated, and polyunsaturated. The classification of fatty acids is shown in Figure 1. And there is a fourth artificial subset, trans fats, which is created by hydrogenation [4]. Polyunsaturated fats are further classified into 2 subsets by the first double bond: omega-3 fatty acids and omega-6 fatty acids. n-3 PUFAs have the first double bond at the third carbon from the methyl terminal, whereas omega-6 polyunsaturated fatty acids (n-6 PUFAs) have the first double bond at the sixth carbon [5]. Mammalian cells are short of the desaturase that can convert n-6 to n-3 PUFAs, which means that n-3 PUFAs must be supplied with the diet. Fish, such as mackerel, salmon, sardines, halibut, herring, and tuna, in the human diet is the major source of n-3 PUFAs, containing docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Quite a few kinds of vegetables and vegetable oil, such as flaxseeds, canola, pumpkin seeds, flaxseed oil, canola oil, and perilla seed oil, also can provide n-3 PUFAs, such as

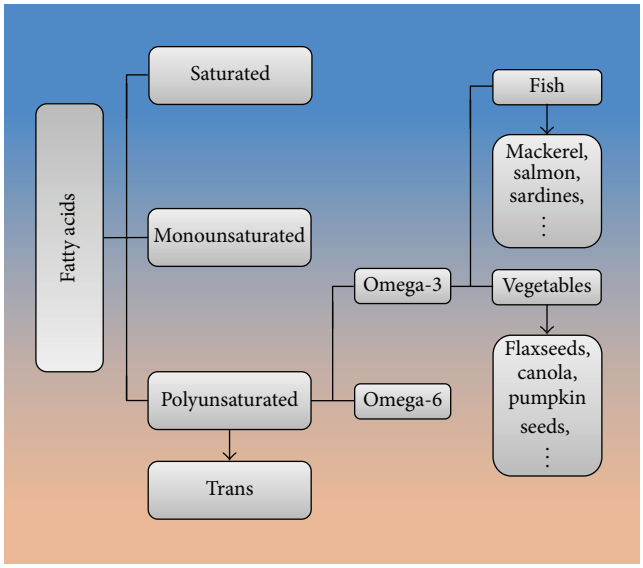

Figure 1: The classification of fatty acids.

alpha-linolenic acid (ALA), which can be converted to EPA and further to DHA by a desaturase enzyme [6]. Isotope-labeled ALA trials suggested that the conversion of natural ALA to EPA is between 0.2% and 21% and further to DHA is between 0% and 9% [7]. The conversion of ALA to DHA and EPA is likely influenced by the competitive inhibition of linoleic acid and negative feedback of DHA and EPA [8]. And the interconversion is limited. So the best way to increase fatty acids intake is to supplement them with specific fatty acids [9].

3. Research Tools for n-3 PUFAs

3.1. Gas Chromatography Methods. All fatty acids in plasma fraction can be analyzed by high performance liquid chromatography and mass spectrometry.

3.2. Fat-1 Transgenic Mice. Mammalian cells are short of the desaturase, which can convert n-6 to n-3 PUFAs [10]. Fat-1 transgenic mice carrying a fat-1 gene expressed a *Caenorhabditis* elegans desaturase that introduces a double bond into n-6 PUFAs to form n-3 PUFAs [11]. Due to the capability, fat-1 transgenic mice are able to produce n-3 PUFAs from n-6 PUFAs and their organs or tissues are rich in n-3 PUFAs without the dietary n-3 PUFAs supplementation [12]. Accordingly, fat-1 transgenic mice can avoid the potential confounding effects from the dietary supplementation [13]. The fat-1 transgenic mice are widely used as new tools for n-3 PUFAs studies.

3.3. Administration Pathway. Rats are chosen in most experimental studies, and some studies also use mice, baboons, and piglets as animal models [14]. The common administration way is oral administration or intragastric administration. The dosage of oral drugs is 0.2 g to 30 g of EPA and DHA/kilogram, and the duration of intervention for studies is from 24 h to 4 weeks [15].

4. n-3 PUFAs and Stroke

4.1. Stroke. Stroke has two main types: ischemic stroke, due to the lack of blood flow, and hemorrhagic stroke, due to the bleeding. Ischemic stroke can be further classified into cerebral infarction and transient ischemic attack (TIA), and hemorrhagic stroke also can be further classified into subarachnoid hemorrhage (SAH) and intracerebral hemorrhage (ICH).

4.2. n-3 PUFAs and Ischemic Stroke. Cerebral infarction is defined as the necrosis of the cerebral tissue caused by ischemia. Under normal circumstances, cerebral blood flow (CBF) is 50 ± 10 mL/100 g/min. When CBF drops to 15 mL/100 g/min, cerebral cortical evoked potential and brain waves disappear completely, but cerebral cells are still alive. And when CBF drops to 8–10 mL/100 g/min, even lower, the function of ion pumps in neuron membrane begins to fail, inducing potassium efflux and sodium influx, and cerebral cells begin to die and cerebral infarction occurs. Traditionally, TIA was defined as the episodes of neurologic dysfunction resulting from focal cerebral ischemia and completely recovers within 24 hours [16]. The American Heart Association renewed the definition in 2009 and changed the definition from time-based to tissue-based [15]. The newest diagnosis of TIA is based on the restricted diffusion on MRI [16]. Currently, the diagnosis of TIA is dependent upon CT or MRI findings heavily. Cerebral ischemia/reperfusion (I/R) injury is a phenomenon that ischemic stroke induces cerebral cells damage, and, after the restoration of hemoperfusion, the ischemic injury even becomes more serious.

Early reperfusion is desirable, but reperfusion also induces additional neural tissue injury and the breakdown of cellular integrity by oxidative stress, excitotoxic signaling, inflammation, and others [17]. Elevated oxidative stress is associated with the pathogenesis of cerebral injury in I/R [18]. During cerebral I/R, the endogenous antioxidative defense systems turn to be ineffective, which results from the inactivation of detoxification systems and the degradation of antioxidants [19, 20]. A multitude of oxygen radicals such as reactive oxygen species (ROS) begin to accumulate and cause apoptosis and cellular damage [21]. ROS are involved in the oxidative damage of proteins, nucleic acids, and lipids in ischemic tissues directly [22]. ROS can also cause lipid peroxidation, which leads to the damage of biological membranes [23]. Classic description of lipid peroxidation mainly contains three steps [24]. First, a hydrogen atom removes from the side chain of polyunsaturated fatty acids, forming the lipid radical. Then the unpaired electron rearranges, forming conjugated dienes. And the lipid radical converts into lipid peroxyl radical by attracting molecular oxygen. Second, the lipid peroxyl radical extracts a hydrogen atom and begins a cycle of peroxidation reaction. Third, two radicals combine and form a nonradical. Beside hydroperoxides, lipid peroxidation also produces aldehydes, lipid hydroxides, and others. The lipid

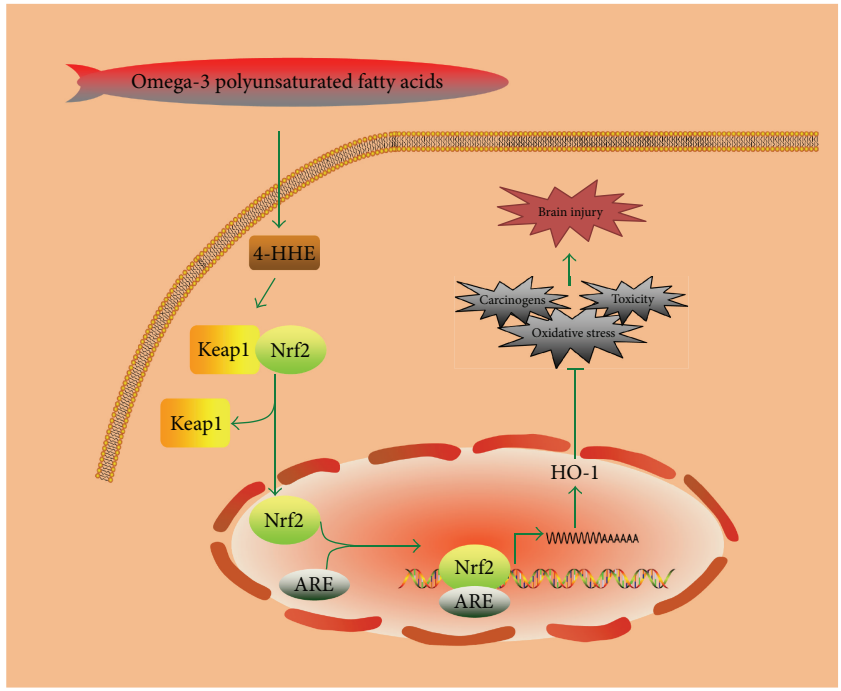

Figure 2: The Nrf2/HO-1 signaling pathway induced by n-3 PUFAs.

peroxidation of n-3 PUFAs is a complex process and ultimately produces the 4-hydroxynonenal. The peroxidation of n-3 PUFAs majorly produces the 4-hydroxy-2E-hexenal (4-HHE); the 4-hydroxy-2E-nonenal (4-HNE) is the major product of n-6 PUFAs, and some other fatty acids produce 4-hydroxy-2E, 6Z-dodecadienal (4-HDDE) [25]. The lipid peroxidation will accumulate these products and affect the normal cell functions, leading to cell death at last. The peroxidation of n-3 PUFAs belongs to nonenzymatic lipid peroxidation, which derived from free radical reactions [26]. The reaction between ROS and transition metals produce the hydroxyl radical, the major radical in this process. The lipid peroxidation will indicate the overproduction of ROS, and this vicious circle may cause the increase of ROS, necrosis, and apoptosis during the time [27]. In addition, the generation of excessive ROS reduces the activation and bioavailability of NO [28]. Oxidative stress and ROS are detrimental factors in the progression of cerebral I/R injury [29]. Oxidative stress can increase the expression of cytokine and the occurrence of edema and apoptosis [30]. During reperfusion, ROS acts as the signaling molecules, inducing the activation of NF-κB and activator protein-1 (AP-1) [31]. Due to the low activities of antioxidant enzymes and the high rates of oxidative metabolic activities, neurons in the brain are more vulnerable to ischemic damage [32]. Free radical generation, calcium overload, excitatory neurotransmitter accumulation, inflammation, and apoptosis are all related to neuronal injuries after ischemic damage [33, 34].

The dietary supplementation of n-3 PUFAs can decrease the volume of cerebral infarction partly by adjusting antioxidant enzymes activities and partly by working as an antioxidant directly [35]. n-3 PUFAs may act as an antioxidant in reducing cerebral lipid peroxides and play a role in regulating oxidative stress through the increase of oxidative burden and the improvement of antioxidative defense capacity [36]. The chronic administration and dietary supplementation of n-3 PUFAs can improve symptoms of cerebral I/R by increasing the antioxidative capacity, as well as reducing the induction of chaperon molecules and the stabilization of membrane integrity and lipid peroxidation [37]. The dietary supplementation of ALA is also found such that it can reduce the level of lipid peroxidation, as well as increasing the risk of spontaneous reperfusion [38]. The neuroprotective effects of n-3 PUFAs include not only inhibiting the oxidative stress but also enhancing the expression of nuclear factor E2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) [39]. The Nrf2/HO-1 signaling pathway induced by n-3 PUFAs is shown in Figure 2. Nrf2/HO-1 signaling pathway is a crucial mechanism of n-3 PUFAs for protecting cells [40]. n-3 PUFAs reduce ischemic injury by activating Nrf2 and increasing HO-1 production [41]. The protective mechanisms are associated with the upregulation of HO-1, the activation of Nrf2, and the oxidation of 4-HHE. 4-HHE is the end-product of n-3 PUFAs by peroxidation and acts as an effective Nrf2 inducer [42]. Nrf2 acts as a transcription factor in regulating phase-2 enzymes expression. Under normal conditions, Kelch-like ECH-associated protein-1 (Keap1), the inhibitory protein of Nrf2, will bind to Nrf2 and lead Nrf2 to the proteasomal degradation process. Under oxidative stress, 4-HHE will react with the cysteine residues of Keap1 and dissociate Nrf2 from Keap1 [41]. Then Nrf2 will translocate into the nucleus, bind to antioxidant responsive element (ARE), and induce the expression of phase-2 enzymes [43]. Phase-2 enzymes like HO-1 mainly mediate the cytoprotection

against oxidative stress, carcinogens, and toxicity. Cerebral ischemia/reperfusion injury will cause the elevated expression of antioxidative proteins and Keap1/Nrf2 system and increase the expression of Nrf2 and HO-1, which can inhibit the activation of microglia and the expression of proinflammatory cytokine [44]. The shortage of Nrf2 or HO-1 will sensitize animals to inflammation and injury induced by ischemic stroke. The neuroprotection against cerebral ischemic stroke mediated by DHA includes improving neuronal defense capacity and inhibiting cellular inflammatory mechanisms by increasing the expression of Nrf2 and HO-1 [45]. Although DHA itself is able to increase the expression of Nrf2 and HO-1 in glial cell cultures, it is not enough to induce the promotion of Nrf2 and HO-1 in vivo. Actually, the treatment with DHA after ischemic stroke only can provide a driving force for the promotion of Nrf2 and HO-1 [46].

On the other side, n-3 PUFAs exhibit the modulatory effects on the homeostasis of redox potential by enhancing the oxidative burden induced by lipid peroxidation and activating the activity of antioxidant enzymes [47]. Under normal conditions, n-3 PUFAs such as DHA may not act as initiators of free radical generation, while in the oxidized environments DHA may augment the oxidative burden. That is to say, n-3 PUFAs treatments may lead to augmenting, or be comparable at least to, the damage induced by cerebral I/R injury [48]. People found that DHA could increase the activity of MPO, the expression of COX-2 mRNA, and the activity of caspase 3, which will exacerbate neurobehavioral deficits and cerebral infarction in the end [49]. The acute posttreatments with DHA after cerebral ischemic stroke are found to augment oxidative burden and subsequently exacerbate cerebral I/R injury remarkably [37]. The detrimental effects of DHA in cerebral I/R are associated with oxidative changes. DHA alone has little effects on the generation of free radical in neuroglia but increases the oxidative burden induced by hydrogen peroxide greatly. The high level of free n-3 PUFAs will induce free radicals to react with unsaturated fatty acids instantaneously [37]. Once beginning, the reaction will continue and propagate an amplification cycle of free radicals generation, which will lead to the augmented oxidative stress and increase the oxidative burden induced by cerebral I/R significantly.

In terms of inflammation, ischemic stroke will trigger complex cellular responses, including the recruitment of inflammatory cells and the activation of glial cells [50]. Leukocytes will move in the interstitial compartments and release the proteolytic enzymes and cytotoxic metabolites, inducing the nerve cells death and enhancing the deleterious effects of ischemic stroke. In the end, leukocytes plugging in capillaries, the aggregation of platelet leukocyte, and the extravasation of albumin occur [51]. n-3 PUFAs are found to inhibit systemic inflammatory responses and modulate vascular inflammation by changing intracellular signal transduction and controlling lipid mediators [52]. The anti-inflammatory effects of n-3 PUFAs include inhibiting the conversion of arachidonate acids to the proinflammatory lipid intermediates, interrupting the NF-κB signaling pathway, and activating the AMP-activated protein kinase, inducing the synthesis of anti-inflammatory lipid mediators like resolvins and protectins [40]. DHA is the precursor of neuroprotectin D1 (NPD1) and NPD1 can downregulate apoptosis, promote neurogenesis, and inhibit leukocyte infiltration and the expression of proinflammatory gene [53]. n-3 PUFAs also exhibit potent immunomodulatory effects by reducing the leukocyte chemotaxis and inhibiting the expression of adhesion molecules [54]. EPA and DHA can exhibit neuroprotective effects through inducing the expression of receptors of chemoattractants and inhibiting the activation of macrophages and microglia and the migration of neutrophils and monocytes. DHA also can increase the generation of antiapoptotic proteins such as Bcl-xL and Bcl-2, which inhibit the inflammatory response mediated by microglial cells [55]. In glial cell culture, DHA exhibits immunosuppressive effects by reducing the phosphorylation of c-Jun N-terminal kinase (JNK) and c-Jun and inhibiting the activation of AP-1 [56]. The activation of JNK plays a crucial role in neuroinflammation and cell death induced by ischemic stroke [57]. Once activated, JNK will increase the phosphorylation of c-Jun, the crucial component of AP-1, and induce the cell death program and transcription-dependent inflammation [58]. The downregulation of JNK/AP-1 signaling pathway, which includes decreasing the phosphorylation of c-Jun and JNK and inhibiting the DNA-binding activity of AP-1, contributes to the neuroprotective effects of DHA against cerebral ischemic stroke [59]. Someone found that G protein-coupled receptor 120 (GPR120) could be activated by long-chain fatty acids and GPR120 acted as a functional receptor or sensor of n-3 PUFAs, exerting the anti-inflammatory effects [60]. Through GPR120, n-3 PUFAs inhibit the activation and phosphorylation of TAK1 by the β-arrestin2/TAB1 dependent effect, resulting in the inhibition of TNF-a and TLR inflammatory signaling pathways [61].

4.3. n-3 PUFAs and Hemorrhagic Stroke. SAH is a pathologic syndrome defined by the appearance of the blood in the subarachnoid space resulting from a wide variety of causes. The most common cause of SAH is trauma, and 85% of nontraumatic patients are in case of underlying cerebral aneurysm [62, 63]; the other 15% are idiopathic [64]. Two-thirds of the idiopathic patients are due to perimesencephalic hemorrhage [65, 66]. According to unenhanced CT, SAH is classified into mainly three distinct forms [67]. Aneurysm rupture and vascular malformation belong to the first form, in which SAH is centered in the central basal or suprasellar cisterns and extends to periphery diffusely [68]. Idiopathic perimesencephalic hemorrhage resulting from aneurysm rupture, vascular malformation, and cervicomedullary junction tumor belong to the second form, in which SAH is centered in the low basal or perimesencephalic cisterns and does not extend. The third form, in which SAH is centered in the cerebral convexities, includes cerebral amyloid angiopathy, reversible cerebral vasoconstriction syndrome, cerebral venous thrombosis, and posterior reversible encephalopathy syndrome. There are several reasons behind the morbidity and mortality of the patients with SAH and cerebral vasospasm (CV) is a significant one of them [69]. The pathogenesis of cerebral vasospasm is still unclear. Inflammation, Endothelin (ET), NO, and products of erythrocyte degradation all have been

confirmed to play crucial roles in CV [70, 71]. OxyHb, produced by erythrocyte degradation, is one of the causes of CV. When blood flows into the subarachnoid space and soak vessels for a long time, dysfunction of vessels occurs and then the blood cells begin to collapse and lipid peroxide and free radicals produced [72]. The products lead to a series of chain reactions, the destruction of biological membrane, the removal of endogenous NO, and the increasing production of ET. The diastolic and systolic function of vessels failed at last, leading to cerebral hemorrhage [73]. Quite a few studies are indicative of the fact that Rho-kinase plays a crucial role in CV [74, 75], and some agents like thromboxane A2 (TXA2) and sphingosylphosphorylcholine (SPC) can activate Rho-kinase [76, 77]. Recently, EPA is reported to inhibit SPC by inducing the activation of Rho-kinase in vitro [78]. Moreover, EPA can change the concentration of arachidonic acid, which has a potential role in CV [79] and inhibit the synthesis of TXA2 [80]. The concentration of free fatty acids increases after SAH and has a secondary elevation between 8 and 10 days after SAH [79]. These observations are suggestive of the fact that EPA can inhibit CV after SAH and improve clinical prognosis by inhibiting the activation of Rho-kinase [81]. Furthermore, oral EPA is found to reduce the risk of CV after SAH [82], and using n-3 PUFAs for immunomodulatory interventions could reduce the risk of delayed cerebral ischemia after SAH [83].

ICH is a pathologic syndrome caused by the rupture of intracranial vessel, which is resulting from nontraumatic factors, and the appearance of the blood in the intracerebral space leads to several causes. Because n-3 PUFAs demonstrate poor effects on ICH, there are a few studies focusing on n-3 PUFAs and ICH. A slice of studies suggests that different concentration of n-3 PUFAs leads to different effects [84]. Low levels of n-3 PUFAs protect against thrombogenesis, while high levels may induce oxidative damage and become a risk factor for ICH [85]. Moreover, high levels may lead to a poor functional outcome and a severe motor impairment after ICH [86].

5. Expectation

Except the antioxidant and anti-inflammatory effects, n-3 PUFAs also can trigger other responses like neuranagenesis and revascularization in stroke. The classification of fatty acids is shown in Figure 1, and the Nrf2/HO-1 signaling pathway induced by n-3 PUFAs is shown in Figure 2. Even though n-3 PUFAs is generally accepted as a beneficial factor in diets, there are still many debates remaining. As most of the previous studies focus on the prevention and post-treatments of stroke, the lack of systematic treatment strategy with n-3 PUFAs remained to be supplemented.

Abbreviations

n-3 PUFA: Omega-3 polyunsaturated fatty acids
N-6 PUFA: Omega-6 polyunsaturated fatty acids
DHA: Docosahexaenoic acid
EPA: Eicosapentaenoic acid
ALA: Alpha-linolenic acid
TIA: Transient ischemic attack
SAH: Subarachnoid hemorrhage
ICH: Intracerebral hemorrhage
CBF: Cerebral blood flow
I/R: Ischemia/reperfusion
ROS: Reactive oxygen species
4-HHE: 4-Hydroxy-2E-hexenal
4-HNE: 4-Hydroxy-2E-nonenal
4-HDDE: 4-Hydroxy-2E, 6Z-dodecadienal
AP-1: Activator protein-1
Nrf2: Nuclear factor E2-related factor
HO-1: Heme oxygenase-1
Keap1: Kelch-like ECH-associated protein-1
ARE: Antioxidant responsive element
NPD1: Neuroprotectin D1
JNK: c-Jun N-terminal kinase
GPR120: G protein-coupled receptor 120
CV: Cerebral vasospasm
ET: Endothelin
TXA2: Thromboxane A2
SPC: Sphingosylphosphorylcholine.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Jiyuan Bu and Yang Dou contributed equally to this work.

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Research Article

Chronic Alcohol Intoxication and Cortical Ischemia: Study of Their Comorbidity and the Protective Effects of Minocycline

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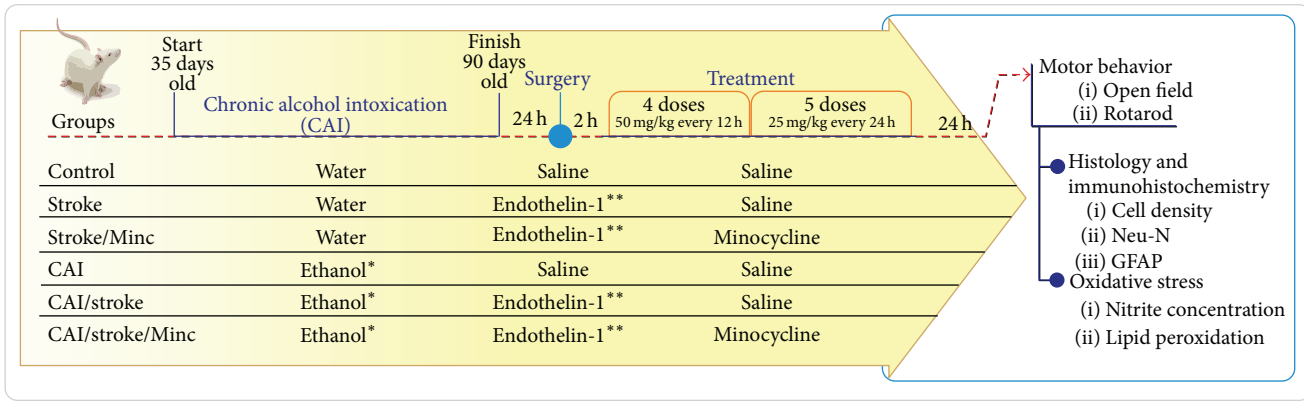
Chronic alcohol intoxication (CAI) increases both morbidity and mortality of stroke patients. Despite the high prevalence of CAI and ischemic stroke, studies addressing their comorbidity and/or protective alternatives remain scarce. Thus, the influence of CAI on both stroke outcome and minocycline treatment (recognized for its neuroprotective effect) was investigated. Female Wistar rats (35 days old) were treated with water or ethanol (6.5 g/kg/day, 22.5% w/v) for 55 days. Then, focal ischemia was induced by endothelin-1 in the motor cortex. Two hours later, four doses of 50 mg/kg of minocycline every 12 hours followed by five doses of 25 mg/kg every 24 hours were administered. Behavioral performance (open field and rotarod tests) and immunohistochemical (cellular density, neuronal death, and astrocytic activation) and biochemical (lipid peroxidation and nitrite levels) analyses were performed. CAI increased motor disruption, nitrite and lipid peroxidation levels, and neuronal loss caused by ischemia, whereas it reduced the astrogliosis. Minocycline was effective in preventing the motor and tissue damage caused by stroke. However, these effects were attenuated when CAI preceded stroke. Our data suggest that CAI beginning in adolescence contributes to a worse outcome in ischemic stroke survivors and reduces the benefits of minocycline, possibly requiring adjustments in therapy.

1. Introduction

Heavy alcohol drinking significantly increases both morbidity and mortality of stroke patients [1]. It is also a major risk factor for cerebrovascular diseases [1]. Female and young adults have been highlighted as major groups with the highest growth in alcohol consumption for the next decades [2]. In Brazil, female population was already pointed as the group with the largest increase in ethanol consumption during the period 2006–2012 [3]. Chronic alcohol intoxication (CAI) usually begins during adolescence triggering neuroinflammatory and oxidative processes, leading to significant neuronal loss (these mechanisms are also shared by ischemic stroke) [4]. Despite the high prevalence of CAI and ischemic stroke found in clinical practice, few preclinical and clinical studies have addressed the consequences of their comorbidity.

Our preliminary results revealed motor deficits and increased microglial activation in ischemic rats previously intoxicated with ethanol [5], leading us to ask about the role of



*6.5 mg/kg/day
**40 pmol/1 μL
Minc: minocycline
CAI: chronic alcohol intoxication

Figure 1: Experimental design.

oxidative stress in the comorbidity. Moreover, treatment with minocycline was able to partially prevent these alterations. Recent studies proposed minocycline as a promising therapy for stroke. Clinical improvement of the neurological outcomes of the patients was observed after 30 days of treatment with this drug [6]. However, to date no study has showed the possible role of oxidative stress in the alterations of such response in the comorbidity with CAI.

Minocycline is a potent anti-inflammatory/antibiotic tetracycline with well-established neuroprotective effects [7, 8]. In the present study, we investigated the putative neuroprotective effects of minocycline treatment in ischemic rats chronically intoxicated with ethanol since adolescence, with a special focus on the analysis of glia activation and oxidative stress.

2. Materials and Methods

2.1. Animals and Ethical Aspects. Sixty female Wistar rats (35 days old) were housed (21 ± 2°C; 12 h light/dark cycle) with food and water *ad libitum*. All efforts were carried out to reduce the number of animals and minimize their suffering. This study followed the NIH Guide for the Care and Use of Laboratory Animals and it was approved by the Committee for Ethics in Experimental Research with Animals of the Federal University of Pará (license number BIO007-09).

2.2. Study Design. Animals were randomly divided into six groups (10 animals/group) (Figure 1). They received orally (gavage) distilled water or ethanol (6.5 g/kg/day, 22.5% w/v) once a day, for 55 days (animals were 90 days old at the end of this treatment). Then, focal ischemia was induced by stereotaxic microinjection of 1 μL (40 pmol) endothelin-1 (ET-1), a potent vasoconstrictor, into the left motor cortex (2.3 mm lateral and 1.2 mm posterior from bregma and 0.4 mm below the pial surface).

Two hours after ischemia induction (counted after the removal of the cannula), animals received saline or minocycline intraperitoneally (four doses of 50 mg/kg every 12 hours followed by five doses of 25 mg/kg every 24 hours). The latter treatment was according to that previously described [11].

No death was observed within the 55 days of the ethanol treatment as well as after the treatments with ET-1 and/or minocycline (data not shown). All the behavioral, histopathological, immunohistochemical, and biochemical analyses were performed by an experienced experimenter who was unaware of the experimental group of the animals tested.

2.3. Behavioral Assays. Twenty-four hours after the last drug administration, animals were acclimated for 1 h in a room with attenuation of noise levels and low illumination (12 lux). Then, the open field (5 min) and the rotarod (three successive trials of 3 min each one, on the rotating rod at 15 rpm) tests were carried out [5].

2.4. Biochemical Analysis. After behavioral assays, five animals per group were sacrificed by cervical dislocation and cerebral cortex was collected and processed for spectrophotometric analysis of lipid peroxidation (LPO, using malondialdehyde (MDA) as an indicator) and nitrite levels (an indirect marker of nitric oxide production), as previously described [12, 13]. Data were corrected according to the protein concentration of each sample [14]. Then, results were expressed as percentages of control groups.

2.5. Histological and Immunohistochemical Evaluations. Five animals per group (different from those for biochemical analysis) were deeply anesthetized (ketamine) and transcardially perfused with a solution of 4% paraformaldehyde diluted in 0.2 M phosphate buffer. Frozen sections of postfixed brains were stained with cresyl violet and they were analyzed as previously described [5, 15].

Coronal sections of cerebral cortex were also analyzed for Neu-N-positive neurons and GFAP-positive astrocytes by immunohistochemistry (1 : 500 and 1 : 1000, resp.). Briefly, slide-mounted sections were kept in an oven at 37°C for 30 min and rinsed once in 0.1 M phosphate buffer saline (PBS)

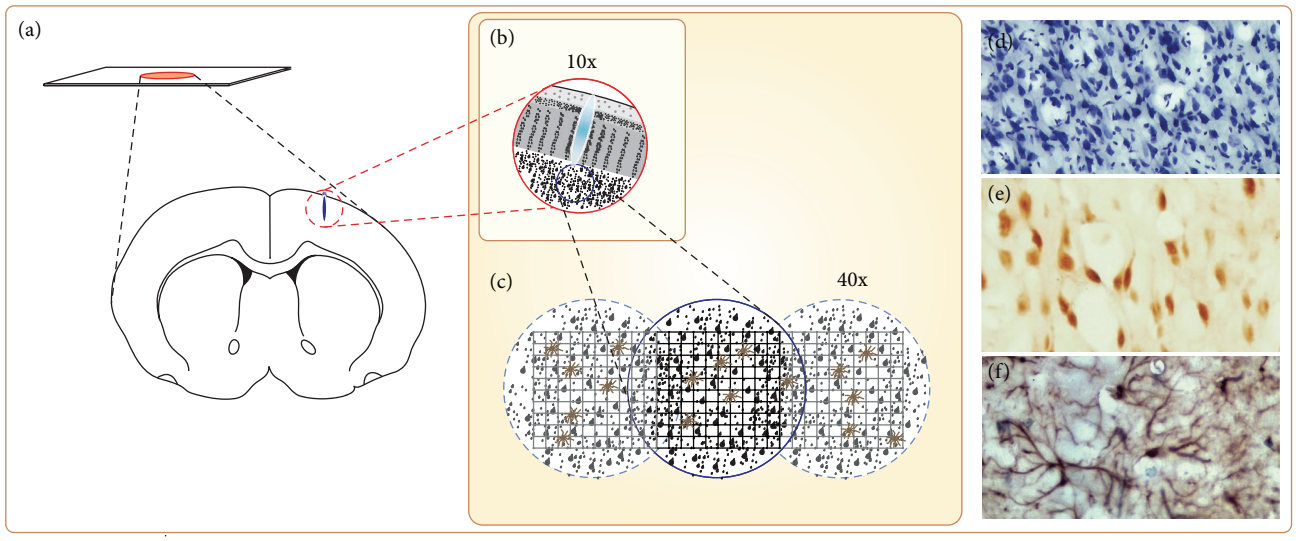


Figure 2: Method for histological and immunohistochemical evaluations in motor cortex. (a) Schematic representation of the brain slices; (b) 10x magnification; (c) 40x magnification; (d) cresyl violet-stained cells; (e) Neu-N positive cells; (f) GFAP-positive cells.

for 5 min. To improve labeling intensity, sections were then pretreated in 0.2 M boric acid (pH 9.0) previously heated to 65°C for 25 min. Sections were further allowed to cool for about 20 min and incubated under constant agitation in 1% hydrogen peroxide solution in methanol for 20 min. Sections were then rinsed in 0.05% PBS/Tween (Sigma, USA) solution for 3 min (three times) and incubated with 10% normal horse (for anti-Neu-N antibody) or normal goat (for anti-GFAP antibody) serum in PBS for 30 min. Sections were then incubated with the primary antibody diluted in PBS for 2 h, rinsed in PBS/Tween solution for 3 min (3 times), and incubated with the biotinylated horse anti-mouse (anti-Neu-N antibody) or goat anti-rabbit (anti-GFAP antibody) secondary antibodies (Vector Laboratories, USA) diluted at 1 : 100 and 1 : 200, respectively, in PBS for 1 h. Sections were washed three times and incubated in the complex avidin-biotin-peroxidase (ABC Kit, Vector Laboratories, USA) for 45 min. Sections were then rinsed four times and DAB-reacted according to the protocol described elsewhere [5, 15]. After DAB reaction, sections were rinsed three times in 0.1 M phosphate buffer, dehydrated using alcohols and xylene, and covered with a coverslip.

For quantitative assessments, the number of Neu-N-positive neurons and GFAP-positive astrocytes was evaluated by using a square 0.25 mm wide grid in the eyepiece of the microscope (Figure 2). This grid corresponds to an area of 0.0625 mm^2. At least, three fields in the motor cortex per section and three sections per animal of each group were analyzed.

2.6. Statistical Analysis. All values were expressed as means ± SEM. Gaussian distribution was analyzed by Kolmogorov-Smirnoff test. Two-way analysis of variance (ANOVA) followed by *post hoc* Bonferroni's test was applied. Level of significance was set at $P < 0.05$.

3. Results

Chronic alcoholic intoxication (CAI) exacerbated the motor deficits induced by motor cortex ischemia in rats. CAI/stroke group showed high latency to initiate movements, low number of rearing times, and reduced distance travelled in the open field (Figure 3(a)). Also, an increase in the number of falls in rotarod test was detected for this group (Figure 3(b)).

Both CAI and ischemia increased *per se* the levels of both nitrite (Figure 3(c)) and LPO (Figure 3(d)) in cerebral cortex. A synergistic response was observed in the CAI/stroke group (Figures 3(c) and 3(d)).

CAI did not significantly worsen the decrease in the number of cells caused by ischemia (Figure 4(a)), but it diminished the number of neuronal (Neu-N-positive) cells (Figure 4(b)) in the motor cortex. Both CAI and ischemia individually enhanced astrocytic (GFAP-positive) activation (Figure 4(c)). Interestingly, CAI attenuated the ischemia-induced increase of astrocytic activation (Figure 4(c)).

Treatment with minocycline counteracted the motor impairment induced by CAI, ischemia, and their association in open field (Figure 5(a)) and rotarod (Figure 5(b)). Although minocycline was effective and it prevented oxidative stress caused by ischemia, it was only partially effective for reducing nitrite and MDA levels in the ischemic rats previously intoxicated with ethanol (Figures 5(c) and 5(d)).

Finally, CAI did not modify the protective effects of minocycline and it prevented both the ischemia-induced cellular death (Figure 6(a)) and astrocytic activation (Figure 6(c)). Nonetheless, the previous chronic ethanol intoxication reduced significantly the protective effects of minocycline against the neuronal loss caused by ischemia (Figure 6(b)).

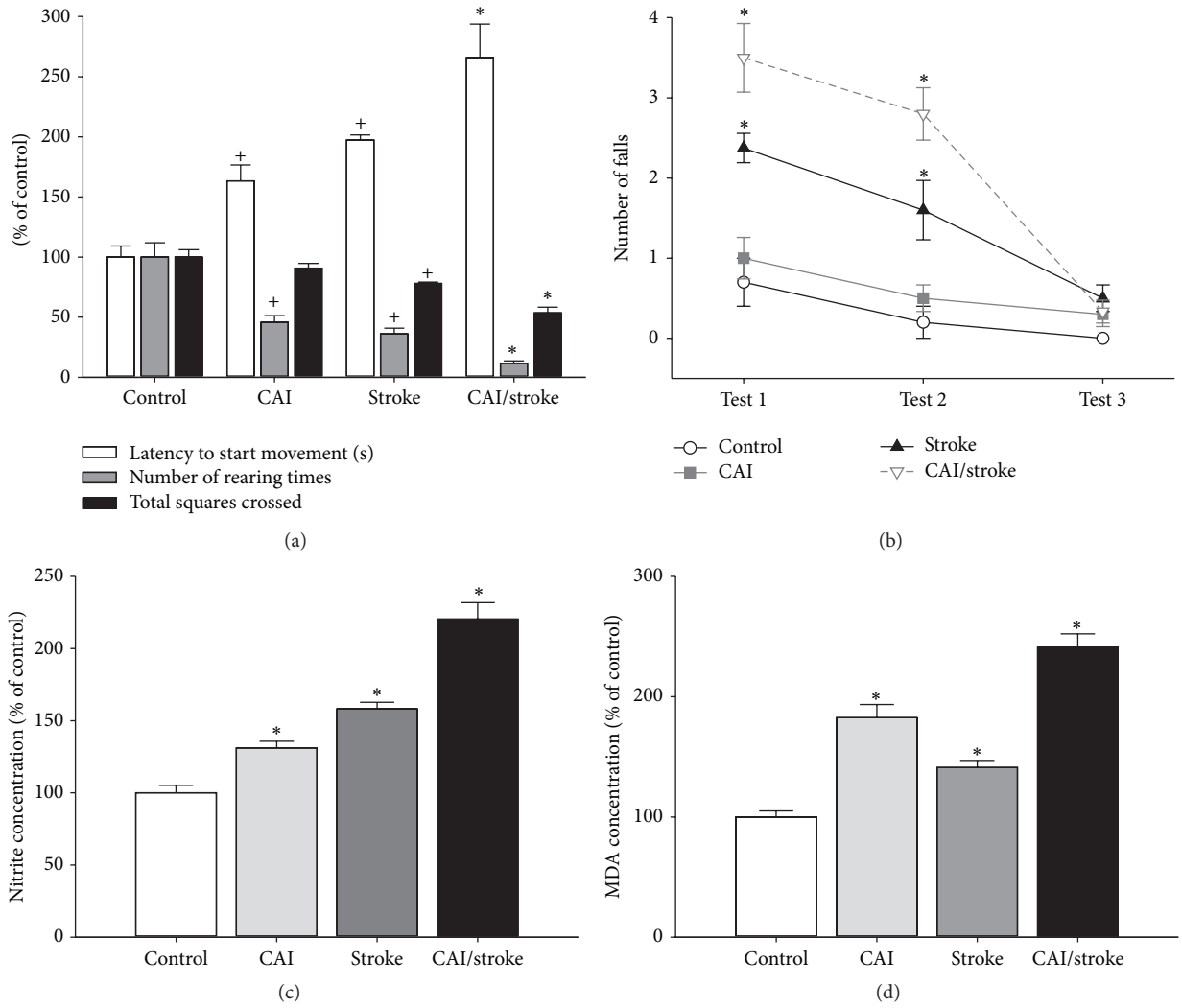


Figure 3: Chronic alcohol intoxication (CAI) worsened the ischemia (stroke) motor outcome and oxidative stress in motor cortex. (a) Open field; (b) rotarod (three sessions at 15 rpm); (c) nitrite concentration; (d) lipid peroxidation (malonaldehyde (MDA) concentration). Data: mean ± SEM [$n = 10$ (a, b), $n = 5$ (c, d)]. $^{*}P < 0.05$ versus all groups; $^{+}P < 0.05$ versus control.

4. Discussion

The heavy ethanol consumption has been epidemiologically highlighted as an independent risk factor for the prevalence and mortality of stroke patients [16]. However, preclinical studies about their comorbidity and/or its consequences for the therapy efficacy are scarce.

Our model of CAI (heavy and regular ethanol intake from adolescence to adulthood) reproduces the largest increase in ethanol consumption of human during a critical period of neurodevelopment [17]. Also, we used females because the last data in Brazil during the period 2006–2012 have pointed to female group as the group with the largest increase in ethanol consumption [3].

Our previous results showed that the same CAI protocol leads to motor and memory impairments in rats, accompanied by a marked neuronal death and reduced microglial and astrocytic densities in both hippocampus and motor cortex [9, 10]. These deleterious consequences were more prominent in cerebral cortex with a major role for the increased oxidative stress (Figure 7).

Motor cortex is one of the main brain areas responsible for the clinical manifestations in stroke outcome (up to 42% of stroke survivors showed motor impairments with alterations of voluntary movements, closely associated with cortical function) [18]. Our results may indicate that ethanol intoxication would already induce significant motor changes as a starting point for a possible synergism in the comorbidity with stroke. Our preliminary results with the current model [5] highlighted this hypothesis revealing the development of motor incoordination and locomotor activity deficits associated with intense microglial activation in ischemic rats previously intoxicated with ethanol. Thus, this study advanced the investigation of CAI-ischemia interactions in

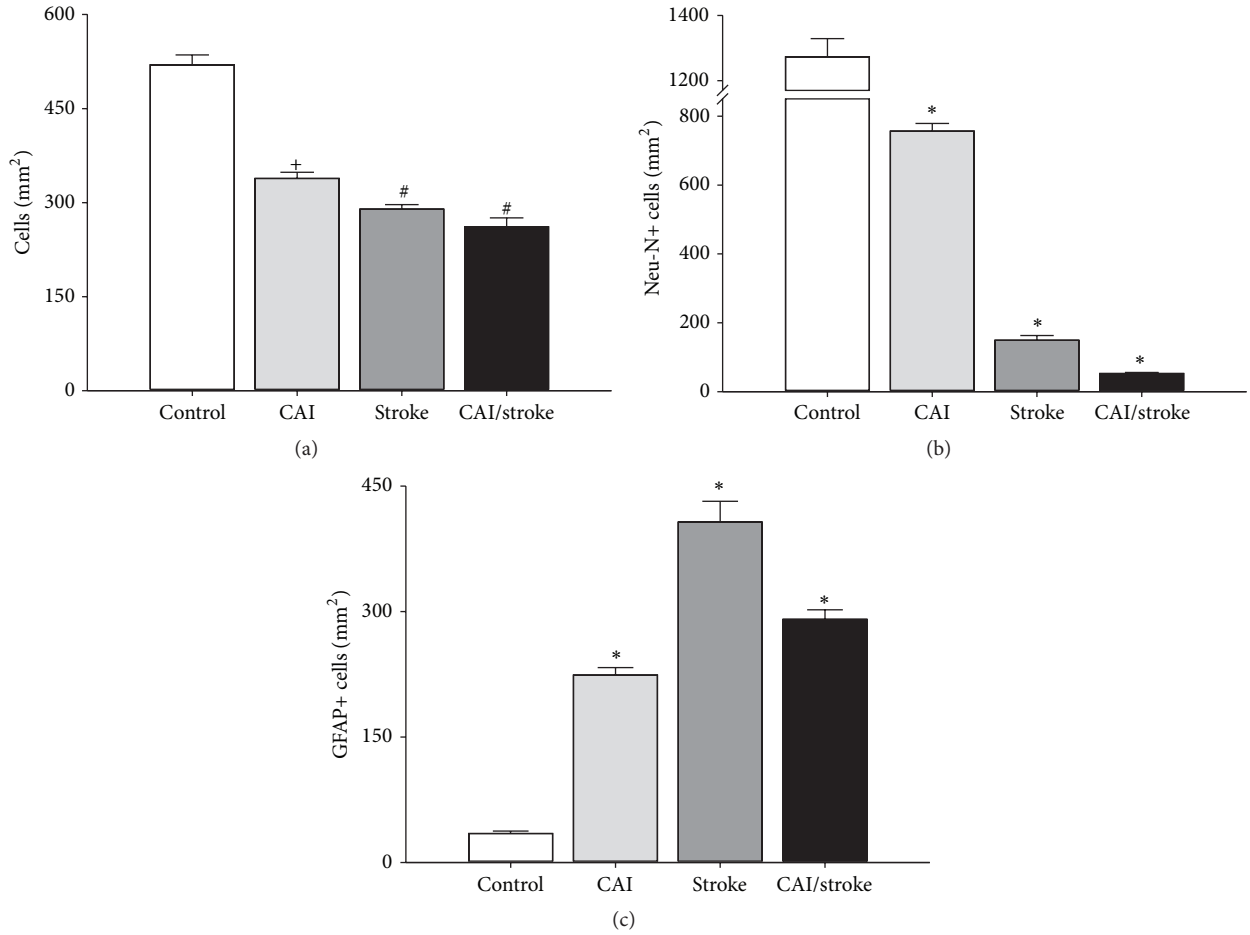


Figure 4: Chronic alcohol intoxication (CAI) increases neuronal loss and astrocytic activation caused by ischemia (stroke). (a) Total number of cells; (b) Neu-N-positive cells; (c) GFAP-positive cells. Data: mean ± SEM ($n = 5$). $^{*}P < 0.05$ versus all groups; $^{+}P < 0.05$ versus control; $^{\#}P < 0.05$ versus control and CAI.

three different levels (behavioral, cellular, and biochemical) with a special focus on the analysis of oxidative stress.

Focal ischemia in the motor cortex region decreased the spontaneous locomotor activity of rats in the open field and induced intense impairments of coordination, balance, and motor learning in the rotarod. These events were observed seven days after the ischemic induction, so they probably result due to the expansion of the ischemic core and penumbra areas after stroke, damaging the connection of cortex with striatum and cerebellum [19].

The current findings corroborate earlier results suggesting motor disruption in animals with endothelin-1-induced focal ischemia [20]. These results also agree with the clinical occurrence of most frequent manifestations in stroke survivors [21].

These behavioral alterations were accompanied by the exacerbated oxidative stress process (as revealed by high MDA and nitrite levels, with increases of about 40% and 60%, resp.) in cerebral cortex. In ischemic injury, oxidative stress is mainly triggered by glutamatergic excitotoxicity as a result of the activation of numerous calcium-dependent enzymes (especially neuronal nitric oxide synthase (nNOS)) that produce excessive amounts of oxygen and other reactive species. Later, inducible NOS (iNOS) contributes to high production of nitric oxide that plays an important role in neurodegeneration of the ischemic penumbra area. Also, increased production of superoxide radicals, by the action of xanthine oxidase (OX) and NADPH oxidase (NOX), takes place, primarily in glial cells and leukocytes [22].

In a similar way to that observed for focal ischemia, CAI also disrupted motor performance with a significant exacerbation of oxidative stress (increases of about 80% and 30% for MDA and nitrite levels, resp.). These results reinforce the involvement of ethanol in the production of reactive oxygen ($O_2^{\bullet}$ and $OH^{\bullet}$, among others) and nitrogen (especially nitric oxide) species, peroxidation and fragmentation of macromolecules, mitochondrial damage, and neurodegeneration [23]. Ethanol also increases the activity of NOX, a protein complex responsible for the "respiratory burst" in phagocytic cells, generating large amounts of reactive species [24]. Other mechanisms involved in this process are the increase of phospholipase A2 activity (leading to oxidation,

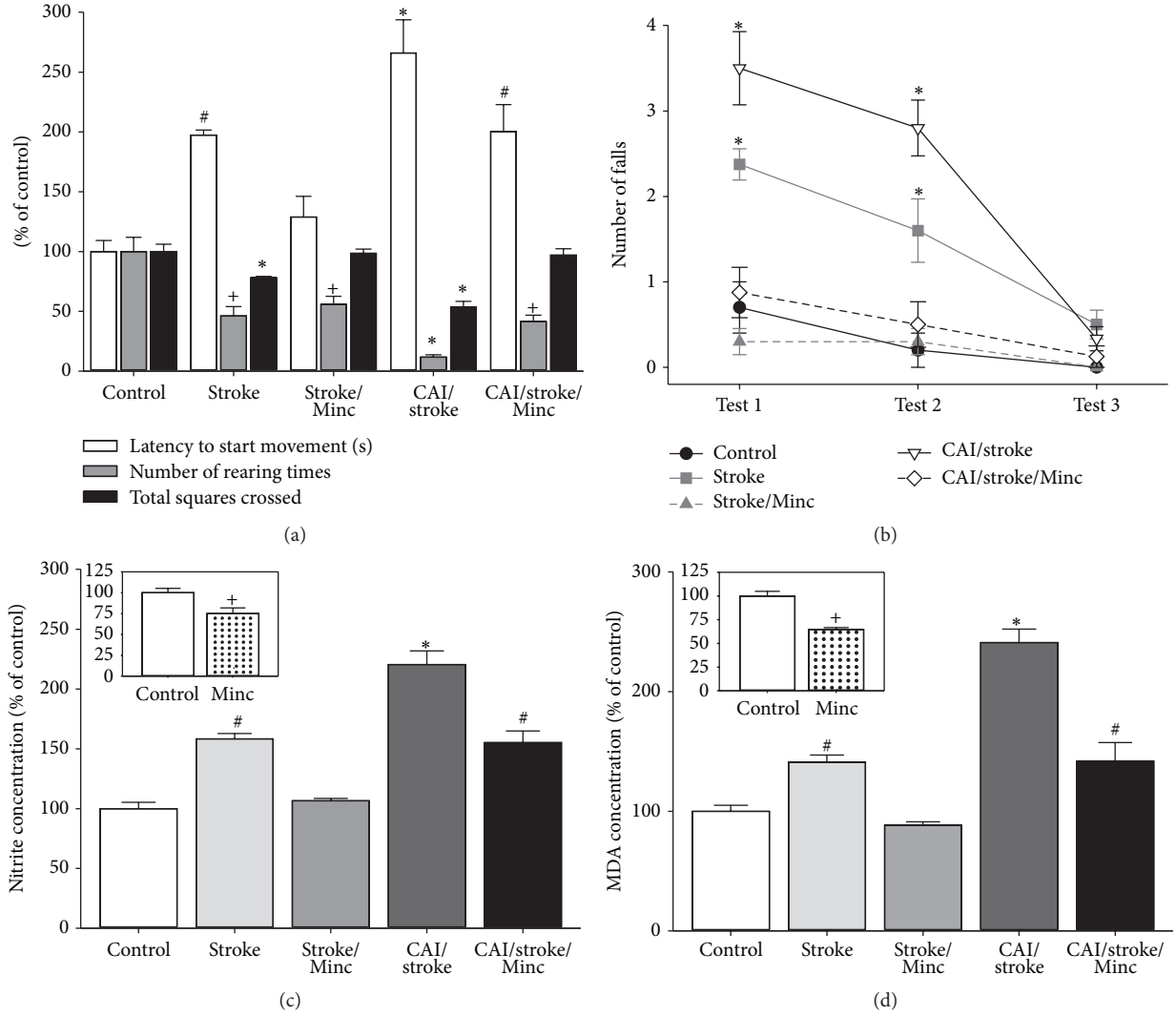


Figure 5: Chronic alcohol intoxication (CAI) reduces minocycline- (Minc-) induced protection in behavioral alterations and oxidative stress caused by ischemia (stroke). (a) Open field; (b) rotarod (three sessions at 15 rpm); (c) nitrite concentration; (d) lipid peroxidation (malonaldehyde (MDA) concentration). Data: mean ± SEM [$n = 10$ (a, b), $n = 5$ (c, d)]. $^{*}P < 0.05$ versus all groups; $^{+}P < 0.05$ versus control; $^{\#}P < 0.05$ versus control and stroke/Minc.

peroxidation, and epoxidation of fatty acids), oxidation of CYP2E1, deficits in removal mechanisms of reactive species, and depletion of endogenous antioxidants [25, 26].

Because the behavioral and neurochemical assessments were performed eight days after the last ethanol administration, a possible influence of a withdrawal syndrome cannot be ruled out. The long-term inhibitory effects of ethanol on glutamatergic neurotransmission can lead to adaptive changes including the upregulation of glutamate receptors and glutamate excitotoxicity during withdrawal period (that causes the breakdown of cytosolic calcium balance and oxidative stress). Thus, this aspect of the experimental design can be translated into an alcoholic patient hospitalized after a stroke.

Comparing our results with the previous data, 24 hours after the end of CAI, higher increase of nitrite levels (70%), but similar increase in LPO (80%), was observed in cortex ([9] and this work). Similar levels of LPO after eight days without ethanol intake, despite the decreased nitrite concentrations, suggest that, in addition to nitric oxide, other reactive species and mechanisms common for both alcoholism and abstinence (increased $O_2^{\bullet}$ and $OH^{\bullet}$, NOX, phospholipase induction, etc.) could be playing an essential role in the deleterious consequences of oxidative stress [23].

The current findings demonstrate for the first time that CAI induces a deleterious synergism effect with stroke in behavioral and biochemical parameters.

Ischemia decreased cellular density and exacerbated neurodegeneration (loss of 88% of Neu-N-positive cells) in the cerebral cortex. These results are in accordance with previous data showing high neurodegeneration after seven days of the tissue infarction [19]. CAI, followed by eight days of

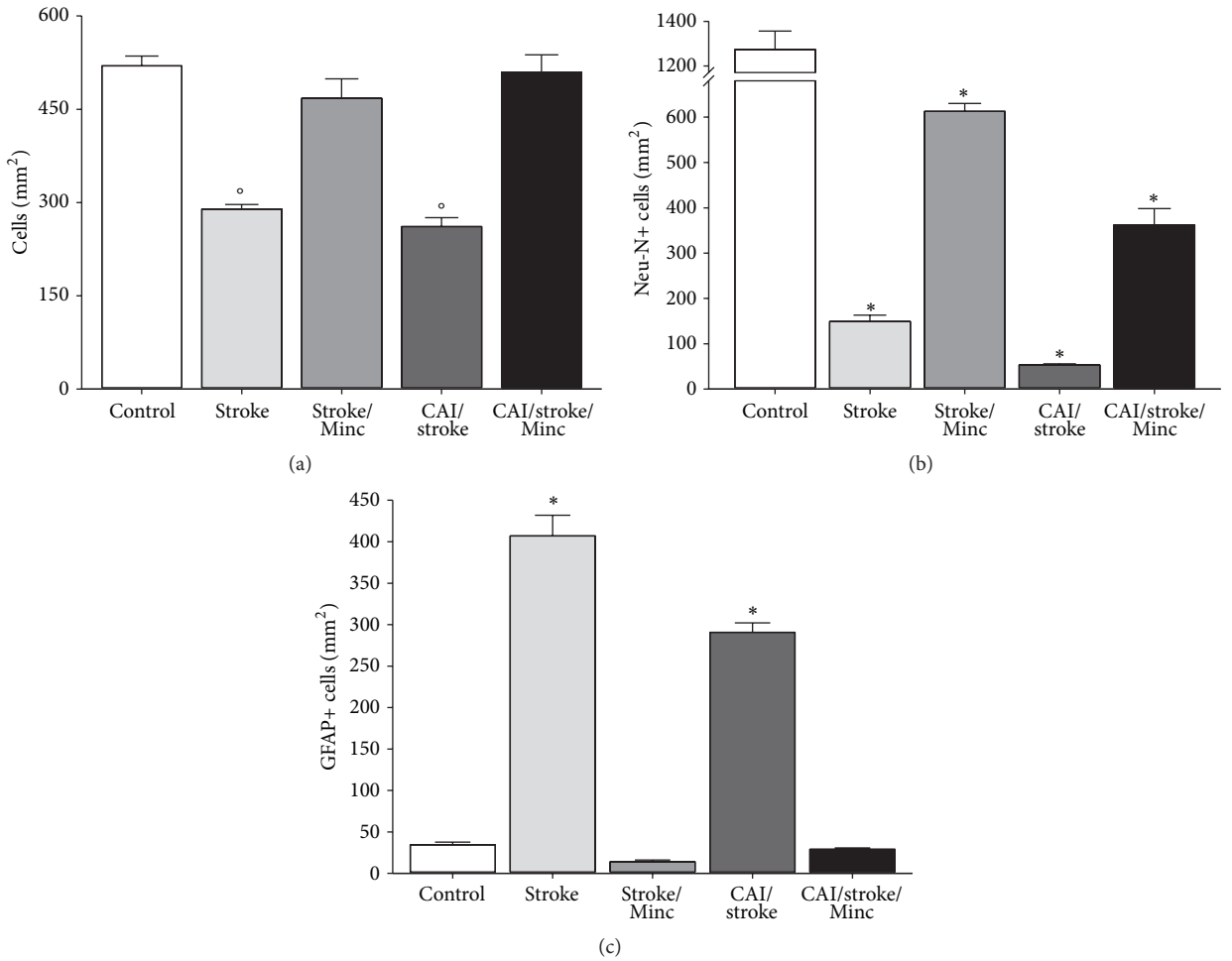


FIGURE 6: Chronic alcohol intoxication (CAI) limited beneficial effects of minocycline (Min) on neuronal loss and astrocytic activation caused by ischemia (stroke). (a) Total number of cells; (b) Neu-N-positive cells; (c) GFAP-positive cells. Data: mean ± SEM ($n = 5$). $^*P < 0.05$ versus all groups; $^\circ P < 0.05$ versus stroke, stroke/Minc, and CAI/stroke/Minc.

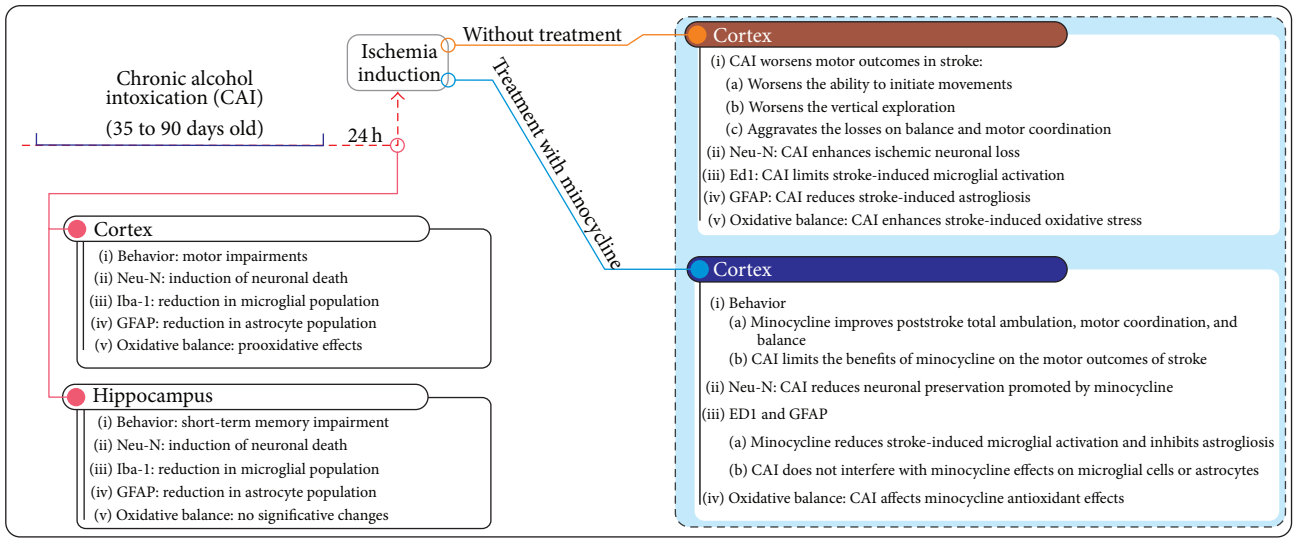


FIGURE 7: Overview of results already observed with this experimental design. Data from this paper and from previously described studies of our group [5, 9, 10].

abstinence, also reduced Neu-N-positive cells in 40%. The same immunostaining in the motor cortex, performed 24 h after the end of CAI, revealed about 40% of neuronal loss [9]. Therefore, we can hypothesize that the neurodegeneration observed in the present study is mainly due to CAI protocol with a minor role for the eight days of abstinence. The association of CAI and ischemia increased the loss of neuronal cell bodies, supporting the hypothesis that the synergy observed in both behavioral and oxidative stress analyses underlies an important neurodegenerative component.

Several mechanisms, including glial activation and neuroinflammation, have been proposed to explain the neurodegeneration induced by CAI and ischemia. Glial activation is a key element observed in animals treated with ethanol for 25 days (5 g/kg followed by 3 g/kg every 8 h, alternating 4 days of intoxication with 3 days of abstinence) [27]. However, it is not still clear if this activation is the cause or the consequence of the ethanol-induced neurotoxicity. Inflammatory events during CAI are associated with the activation of Toll-like receptors (TLR2, TLR3, and TLR4) by ethanol, with the consequent nuclear transcription factor kappa B (NF-κB) induction and the increased expression of inflammatory mediators (COX-2, iNOS, TNF-α, IL-1β, and IL-6, among others) [24, 28].

Previously, the reduced microglial density was observed eight days after CAI [5]. Still, the possible withdrawal syndrome does not seem to play a major role in such effect because a similar decrease of microglial activation was detected (using IBA1-positive cells) 24 h after the last ethanol administration [9]. Also, a previous study already demonstrated that CAI could increase the levels of inflammatory mediators (iNOS, COX-2, and IL-1β) in the cerebral cortex and astrocytes culture, with the absence of microglial activation [29]. In accordance with this view, a significant astrogliosis was observed eight days after the end of CAI. However, in this case, alcohol withdrawal period may have influenced this response since astrocytic population was significantly reduced 24 h after the end of CAI [9]. According to this hypothesis, a previous study demonstrated a significant decrease in the number of astrocytes in rats exposed to CAI for 60 days and the occurrence of astrogliosis after three days of abstinence [30].

Stroke significantly increased astrocytic populations. Ischemic damage has been already characterized by the rapid recruitment and activation of astrocytes and microglial cells towards the injury core [31], probably triggered by TLR activation, proinflammatory mediators, and chemotactic factors.

Surprisingly, the association of CAI and ischemia induced significant lower levels of astrocytic activation than those caused by ischemia alone. These findings suggest that ischemia-induced astrogliosis may be limited by ethanol-induced cellular degeneration before the stroke [9].

The increased proliferation and reactivity of astrocytes play an important role in limiting the damage associated with focal ischemia. Spontaneous recovery after ischemia is directly associated with the formation of an astroglial scar (accumulation of these cells in the perinuclear region), which prevents its expansion [32]. A reduced astrocytic population could therefore influence the progression of the ischemic process by delaying or attenuating the formation of the glial scar. This would also affect the neurodegeneration in the penumbra region and the behavioral outcome.

Moreover, CAI affected not only the stroke outcome, but also the response to the therapy with minocycline. Previous studies demonstrated that minocycline is able to cross the blood brain barrier, promoting neuroprotective effects on the central nervous system after i.p. administration [11, 33, 34]. For each insult (CAI or stroke), protective effects of minocycline were more evident in stroke than in CAI (data not shown), perhaps because only 7 days of treatment occurred during the withdrawal syndrome, after 55 days of CAI. In regard to stroke, treatment with minocycline was effective in preventing/reversing deleterious alterations in behavioral and oxidative parameters. Minocycline treatment alone did not alter any behavioral parameter (data not shown) but it displayed a significant antioxidant effect in the motor cortex (reduced MDA and nitrite levels when compared to those of the control). The mechanisms underlying this latter effect may include decrease of ROS release by the cell (an indirect effect of the drug) and/or the direct scavenging of species such as peroxynitrites (minocycline is able to display an efficacy equivalent to alpha-tocopherol) [35]. The reduced tendency of deleterious side effects with minocycline treatment, in addition to the results over stroke and comorbidity, gives support to the therapeutic use of this drug.

In the association of CAI and stroke, minocycline efficacy was also evident for horizontal ambulation (crossed squares) and motor coordination (falls in rotarod). However, only partial efficacy for the latency to start movement, vertical exploration (rearing), and oxidative stress (MDA and nitrite levels) was observed. This suggests that an increased dose of minocycline, longer periods of treatment with this drug, or the additional use of an antioxidant in the therapeutic protocol must be considered for an adequate treatment of the comorbidity.

Despite the intense neuronal death (more than 75%) and the decrease in total number of cells in the motor cortex caused by ischemia, seven days of minocycline treatment was sufficient to normalize the latter parameter and partially prevent neuronal death. Although neuronal death was even more evident in the comorbidity, minocycline still elicited a significant protective effect. Since neurons are very sensitive to deleterious consequences of oxidative stress, the cotreatment with an antioxidant (as proposed above) may enhance the minocycline protective effect on neuronal tissue in comorbidity cases. Interestingly, treatment with minocycline also prevented astrocytic activation and partially protected against microglial activation caused by association of CAI and stroke.

The mechanisms underlying the benefits of minocycline remain unclear. The first and most widely studied pharmacological effect of minocycline has been its ability to inhibit glial activation [36]. This action is directly and selectively focused on microglial activation associated with neuroinflammation development (named type M1, and different than microglial activation type M2, associated with neuroprotection). M1 activation increases activity of NFκB, with a consequent increased expression of TNF-α, interleukin-1β,

IFN-γ, COX-2/prostaglandins, and NOS. By inhibiting this pathway, minocycline reduces the expression of NFκB and the production of inflammatory mediators [37].

Also, minocycline decreases lesion area and neuronal loss by inhibiting matrix metalloproteinase 9 (MMP-9) activity, a zinc-dependent endopeptidase released by neurons [38]. By cleaving structural compounds of the matrix, this enzyme contributes to worsening the excitotoxicity process, neuronal damage, and cell death, affecting the blood brain barrier integrity.

5. Conclusion

The present study reinforces and extends previous evidence that CAI can exacerbate the deleterious effects of stroke, not only in behavior outcomes but also in altering cellular (worsening neuronal death and astrocytic activation) and biochemical responses (with a synergic deleterious effect in oxidative stress). Moreover, CAI also reduces the benefits of minocycline treatment against the motor impairments, oxidative stress, and neuronal loss induced by ischemia, possibly requiring adjustments in therapy.

Competing Interests

The authors declare that there are no competing interests related to this paper.

Authors' Contributions

Enéas Andrade Fontes-Júnior and Cristiane Socorro Ferraz Maia contributed equally to this work.

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Review Article

The Injury and Therapy of Reactive Oxygen Species in Intracerebral Hemorrhage Looking at Mitochondria

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Intracerebral hemorrhage is an emerging major health problem often resulting in death or disability. Reactive oxygen species (ROS) have been identified as one of the major damaging factors in ischemic stroke. However, there is less discussion about ROS in hemorrhage stroke. Metabolic products of hemoglobin, excitatory amino acids, and inflammatory cells are all sources of ROS, and ROS harm the central nervous system through cell death and structural damage, especially disruption of the blood-brain barrier. We have considered the antioxidant system of the CNS itself and the drugs aiming to decrease ROS after ICH, and we find that mitochondria are key players in all of these aspects. Moreover, when the mitochondrial permeability transition pore opens, ROS-induced ROS release, which leads to extensive liberation of ROS and mitochondrial failure, occurs. Therefore, the mitochondrion may be a significant target for elucidating the problem of ROS in ICH; however, additional experimental support is required.

1. Introduction

Intracerebral hemorrhage (ICH) accounts for 9–27% of strokes worldwide. It is characterized by poor outcomes, with a high mortality rate of 30–50%, and the neurological outcomes of patients who survive are also very poor [1, 2]. The most common cause of intracerebral hemorrhage is hypertension (in approximately 65% of cases), and many other diseases, including amyloid angiopathy, brain tumours, aneurysms, arteriovenous malformations, cerebral cavernous malformations, and arteriovenous fistulae, also contribute to ICH [3].

Until now, there have been no effective medical or surgical therapies to improve outcomes for ICH patients. Therefore, understanding the manner in which ICH induces brain injury is important in the development of effective treatment. In addition to the initial mechanical injury produced by the hematoma, secondary injuries play an important part in further damage [3]. These secondary injuries include not only nerve cell responses to hematoma-induced stress but also the inflammatory reaction caused by the hematoma and the blood coagulation process. In the pathological process of ICH, brain cells, white matter fibre tracts, and the blood-brain barrier (BBB) are injured by the inflammatory reaction. Reactive oxygen species (ROS) are one of the most important components in the inflammatory reaction because they are both products of and participants in the reaction, causing a vicious circle.

Reactive oxygen species (ROS) are created as part of normal cellular metabolism and defence systems. Under physiological conditions, there is a balance between ROS and the antioxidant system; therefore, ROS are regulated by the antioxidant system and kept at a low level. They can take part in many cellular pathways by modulating a number of kinases, phosphatases, redox-sensitive transcription factors, and genes, which contribute to the regulation of cellular growth, differentiation, proliferation, and apoptosis [4]. However, during ICH, there are additional sources of ROS. Greater amounts of ROS can break the dynamic balance between the antioxidant system and ROS, causing cellular injury in the form of lipid peroxidation, DNA damage, and protein oxidation [5]. Therefore, ROS play an important part in the pathophysiology of ICH. ROS can initiate apoptosis and disrupt the blood-brain barrier (BBB), producing damage to the brain [6, 7]. In this review, we will cover current research to understand ICH-related ROS, including their

sources, their injurious effects, their molecular mechanisms, and their relation to the antioxidant system. In addition, we will also summarize therapeutic antioxidant agents and some problems, which may contribute to the development of new therapeutic approaches.

2. Reactive Oxygen Species

Reactive oxygen species are highly reactive and short-lived molecules, including free radicals, such as the superoxide anion radical ($O_2^{\bullet}$) and the hydroxyl radical ($^{\bullet}OH$), and nonradical oxidants, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) [8]. The initial step for ROS production is the univalent reduction of molecular oxygen (O_2) to form superoxide $O_2^{\bullet}$. Under normal physiological conditions, this process is mostly mediated by the mitochondrial electron transport chain (ECT) [9]. Electrons can leak from complexes I and III and are free to react with O_2 to form the superoxide $O_2^{\bullet}$ that is then catalysed by superoxide dismutase (SOD) to form hydrogen peroxide (H_2O_2), which can be processed into the hydroxyl radical ($^{\bullet}OH$) [10]. The ECT, NADPH oxidase, monoaminoxidase, $p66^{shc}$, α-glycerophosphate dehydrogenase, electron transfer flavoprotein (ETF) and ETF dehydrogenase, and aconitase may also contribute to the production of ROS in mitochondria [8]. Normally, most ROS can be neutralized by the antioxidant system to maintain cellular homeostasis. For example, catalase and glutathione peroxidase can convert hydrogen peroxide (H_2O_2) to water. The "residual ROS" are also used as second messengers. They can take part in many cellular processes, such as proliferation and survival; ROS homeostasis and antioxidant gene regulation; mitochondrial oxidative stress, apoptosis, and ageing; iron homeostasis through iron-sulfur cluster proteins; and the ATM-regulated DNA damage response [4]. However, once the balance between ROS and the antioxidant system is broken by some sudden attack, such as ICH, the antioxidant system cannot eliminate the excess ROS, leading to ROS accumulation in the tissue environment. This will damage the mitochondria and lead to additional ROS release, triggering a cascade of damage in the cell.

3. The Sources of ROS in ICH

3.1. Hemoglobin Metabolic Products. After intracerebral hemorrhage (ICH), hematoma and perihematomal regions are rich with RBC lysis products, especially hemin (Figure 1). After intracerebral hemorrhage, red blood cells (RBC), which are present in the hematoma, lyse and release hemoglobin (Hb), which will be degraded to hemin. Hemin can be bound by hemopexin in the serum, and then the complex is transported into the cell via lipoprotein receptor-related protein (LRP1) [11]. Intracellular hemin is degraded into Fe^{2+}, bilirubin, and carbon monoxide (CO). Fe^{2+} derived from hemin can generate a hydroxyl radical, which is the most reactive of all oxygen radicals, via the Fenton reaction, leading to oxidative stress [11, 12]. Hydrocephalus after ICH is also related to iron accumulation [13]. Oxidative stress is very obvious on days 1 and 3 after ICH, but mice pretreated with deferoxamine (DFX) exhibited decreased iron accumulation and neuronal death, attenuated production of reactive oxygen species, reduced microglial activation without affecting astrocytes or neutrophil infiltration, and attenuated white matter damage [14]. Iron regulatory protein-2 (IRP2) showed effects on ferritin expression and then affected iron metabolism and neuronal vulnerability to hemoglobin. Reactive oxygen species formation and heamoxygenase-1 expression after hemoglobin treatment were also attenuated by deletion of the IRP2 gene. These results suggest that IRP2-binding activity increases the vulnerability of neurons to hemoglobin, possibly by reducing ferritin expression [14, 15]. In addition to iron and hemoglobin, bilirubin oxidation products (BOXes) may contribute to ROS release in ICH. Clark et al. reported that production of BOXes via the hemoglobin/Fenton reaction under in vivo conditions has been seen following ICH. In hematomas from a porcine ICH model, the authors observed significant production of BOXes, malondialdehyde, and superoxide dismutase, indicating a potent oxidizing environment [16].

3.2. Excitatory Amino Acids. The initial bleed leads to an influx of glutamate from the bloodstream, and glutamate is one of the most important damaging factors in nervous system, inducing Ca^{2+} overload, which can lead to membrane depolarization and ROS release. Neurons are highly vulnerable to glutamate-induced excitotoxicity. Some evidence shows that glutamate can also participate in brain injury after intracerebral hemorrhage [15]. Activation of the NMDA receptor by glutamate increases Ca^{2+} influx, which mediates an excessive rise in cytosolic Ca^{2+} and consequent mitochondrial Ca^{2+} loading. In addition, during the pathophysiologic process of ICH, production of thrombin after hemorrhage results in activation of Src kinase, which phosphorylates NMDA receptors, enhancing their function [17]. In addition, activation of AMPA receptors also contributes to the influx of Ca^{2+} and Na^{+}, which leads to mitochondrial Ca^{2+} loading, and this process can be blocked by ruthenium red, a mitochondrial calcium uniporter blocker [18]. Mitochondrial Ca^{2+} loading contributes to the decrease in transmembrane potential and the opening of the mitochondrial permeability transition pore (MPTP), causing damage to the mitochondria and mitochondrial respiratory chain and consequent ROS release [19].

3.3. Inflammatory Cells. Microglial activation also contributes to the pathogenesis of brain injury in intracerebral hemorrhage (ICH). Tsirka has reported that inhibiting microglial activation and macrophage infiltration by the tripeptide macrophage/microglial inhibitory factor (MIF) Thr-Lys-Pro attenuated the numbers of ethidium-positive cells compared with saline-treated control mice, reduced production of reactive oxygen species, and improved neurological functional outcomes [20]. In addition, granulocytes can also be a source of ROS after ICH. They can cause the release of ROS via NADPH oxidase and myeloperoxidase [21]. Although these processes are necessary for antimicrobial defence, high ROS levels due to microglial activation and

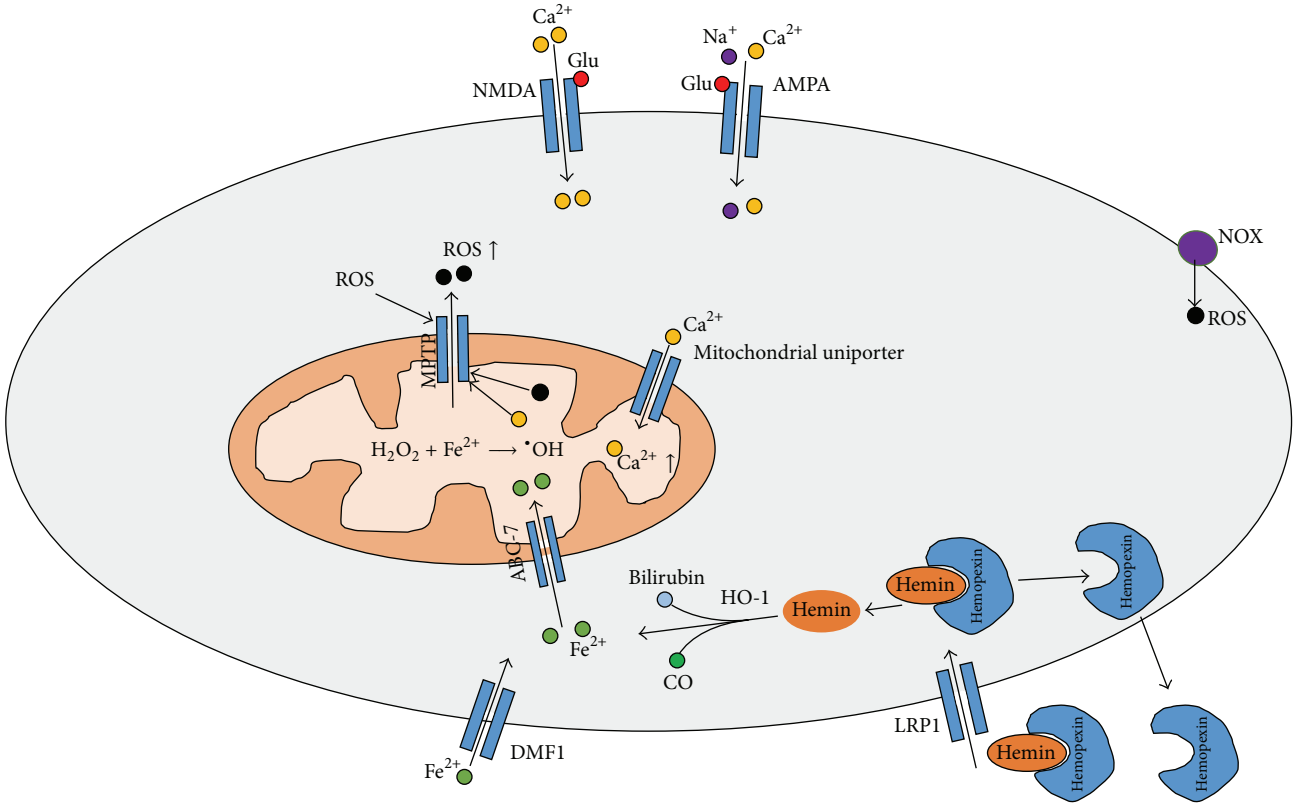

Figure 1: The sources of ROS in ICH: the MPTP can be opened by ROS and Ca^{2+}, followed by ROS release. NMDA receptor activation by glutamate causes cellular Ca^{2+} overload, whereas APMA receptors also contribute to Ca^{2+} overload in the mitochondria. Ferrous iron can be transported into the cell through DMF1 and consequently loaded into the mitochondria by ABC-7; hemin binds with hemopexin and is transported into the cell through LRP1; then, inside the cell, hemin is catalysed by HO-1 into ferrous iron, which is then transported into the mitochondria; ferrous iron is used in the reaction to transform H_2O_2 into the hydroxyl radical, which is a very active radical in oxidative damage. In addition, ROS can also be produced by NOX (ROS: reactive oxygen species; Fe^{2+}: ferrous iron; AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor; $O_2^{\bullet}$: superoxide radical; $^{\bullet}OH$: hydroxyl radical; IRP-2: iron regulatory protein-2; NMDA: N-methyl-D-aspartic acid receptor; fer-1: ferrostatin-1; DMT1: divalent metal transporter 1; HO-1: hemoxygenase-1; MPTP: mitochondrial permeability transition pore; NADPH: adenine dinucleotide phosphate; NOX: adenine dinucleotide phosphate oxidase).

neutrophil infiltration contribute to poor outcomes after ICH [6, 22].

3.4. ROS-Induced ROS Release. The mitochondrial permeability transition pore (MPTP) is a multiprotein complex comprising cyclophilin D, a mitochondrial peptidyl-prolyl cis-trans isomerase; voltage-dependent anion channel (VDAC); adenine nucleotide translocator (ANT); and other molecule(s) that forms a channel in the mitochondrial inner membrane [23]. Its opening plays an important physiological role in maintaining healthy mitochondrial homeostasis (Figure 1). Adaptive and maladaptive responses to redox stress may involve mitochondrial channels such as the mPTP and the inner membrane anion channel (IMAC). The activation of these channels causes intracellular and intramitochondrial redox-environment changes, leading to ROS release. This regenerative cycle of mitochondrial ROS formation and release is named ROS-induced ROS release (RIRR) [8]. At higher levels of ROS, longer mPTP openings may release an ROS burst, leading to destruction of the mitochondrion and, if propagated from mitochondrion to mitochondrion, of the cell itself. Therefore, mitochondria are an important source of ROS. Following ICH, the mPTP was formed, and mROS increased, but these effects could be reversed by the VDAC inhibitor, TRO-19622, or the mROS-specific scavenger, Mito-TEMPO [24].

4. The Contribution of ROS to Brain Injury

4.1. Brain Cell Injury. ROS can cause cellular injury in the form of lipid peroxidation, DNA damage, and protein peroxidation, but organisms also can utilize a series of antioxidant defences, which will be discussed in next section, to protect against oxidative damage [5] (Figure 2). However, ICH-induced ROS are so abundant that antioxidant defences cannot neutralize them, leading to apoptosis through several mechanisms. The classical mechanism is caspase-dependent death: oxidative stress caused by ROS has been shown to induce cytochrome c release, which is often the initiation of apoptosis [6]. ROS can permeabilize the outer mitochondrial membrane, mobilizing cytochrome c from the intermembrane space into the cytosol. Released cytochrome c causes

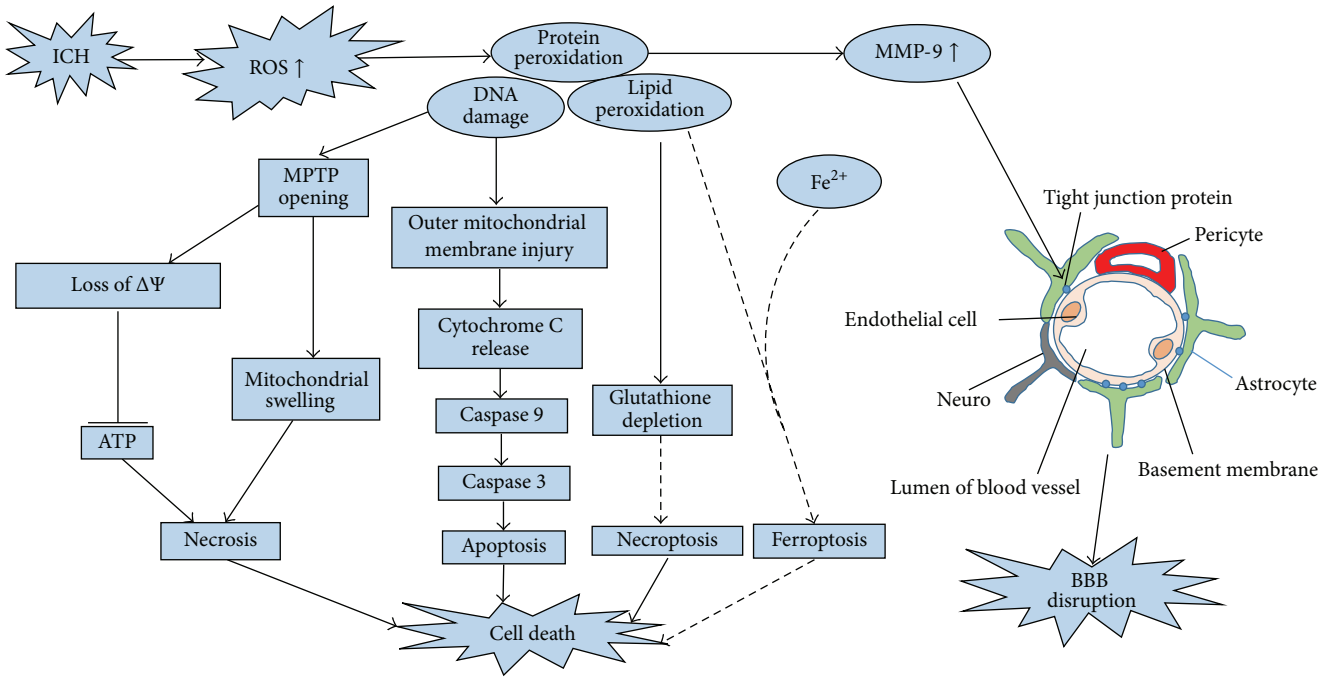

Figure 2: Many additional reactive oxidative species are produced after ICH. They can cause cell death and BBB disruption in the form of lipid peroxidation, DNA damage, and protein peroxidation, which contribute to the opening of the MPTP and to injury of the outer mitochondrial membrane. Once the MPTP opens, many molecules will enter into the mitochondria, and the ΔΨ will decrease, injuring the mitochondrial respiratory chain and causing ATP depletion, causing mitochondrial swelling and necrosis. In addition, ROS can injure the outer mitochondrial membrane, causing cytochrome c release and activating caspase-dependent apoptosis. Excessive ROS can also cause glutathione depletion, leading to necroptosis. Ferroptosis, characterized by iron-dependent accumulation of ROS, may be a potential apoptotic mechanism during ICH. ROS also can upregulate the expression of MMP-9, degrading tight junction proteins and the basal laminar proteins, leading to BBB disruption (ICH: intracerebral hemorrhage; ROS: reactive oxygen species; BBB: blood-brain barrier; MPTP: mitochondrial permeability transition pore; ΔΨ: transmembrane potential; MMP-9: matrix metallopeptidase 9).

formation of the apoptosome and activates initiator caspase 9, which then activates caspase 3, leading to the inevitable onset of apoptotic death [25]. There may be other apoptotic mechanisms in addition to caspase-dependent death. In a simplified in vitro model of hemoglobin neurotoxicity, upstream and downstream caspases were upregulated, but caspase inhibition did not result in neuroprotection, and a free radical scavenger significantly reduced neuronal death, which indicated that another parallel pathway related to oxidative stress may contribute to cell death [26]. Ferroptosis, a newly recognized form of programmed cell death characterized by iron-dependent accumulation of ROS, similar to the pathological process in ICH, may be a potential apoptosis mechanism in ICH [27]. This type of pathway is found in tumour cells, and expression of ferroptosis-related genes, such as lipocalin-2 (LCN2), a protein that participates in iron homeostasis and enhances brain iron clearance after ICH, can be tested in ICH [15]. However, existing evidence for ferroptosis is nonspecific, and more specific biomarkers are needed to characterize the pathway in ICH. Necroptosis is a type of programmed cell death that has been found in the pathological process of ICH [28, 29]. It is characterized by defined molecular mechanisms and can be inhibited by necrostatin-1. Reactive oxygen species are also involved in the regulation of necroptosis, and glutathione depletion may play a role in necroptotic astrocyte injury after ICH [30]. The current minireview discusses the evidence for and against a role for reactive oxygen species in necroptosis. In addition, activation of the mPTP, a pore channel in the mitochondrial membrane, may also be a potential mechanism for necrosis and apoptosis. ROS produced during ICH can attack the mPTP. Once the mPTP opens, it will allow water, large molecules, and iron ions to enter into the mitochondrial matrix, leading to impairment of the mitochondrial respiratory chain (MRC), which results in a greater release of mitochondrial reactive oxygen species (mROS) and cell death [8]. This process has been demonstrated in ischemic stroke, but there is little research about MPTP in ICH. In an ICH mouse model, Ma et al. proved that, following ICH, MPTP is activated, causing an increase in mROS; however, the activator of the MPTP was not mentioned. Therefore, more evidence is needed to demonstrate that the MPTP can be activated by ROS after ICH.

4.2. Injury to the BBB. The blood-brain barrier (BBB) is a dynamic interface between the peripheral circulation and the central nervous system (CNS) that prevents toxic substances from entering into the CNS and contributes to the maintenance of brain homeostasis [31]. The BBB is formed by capillary endothelial cells, which are connected by tight

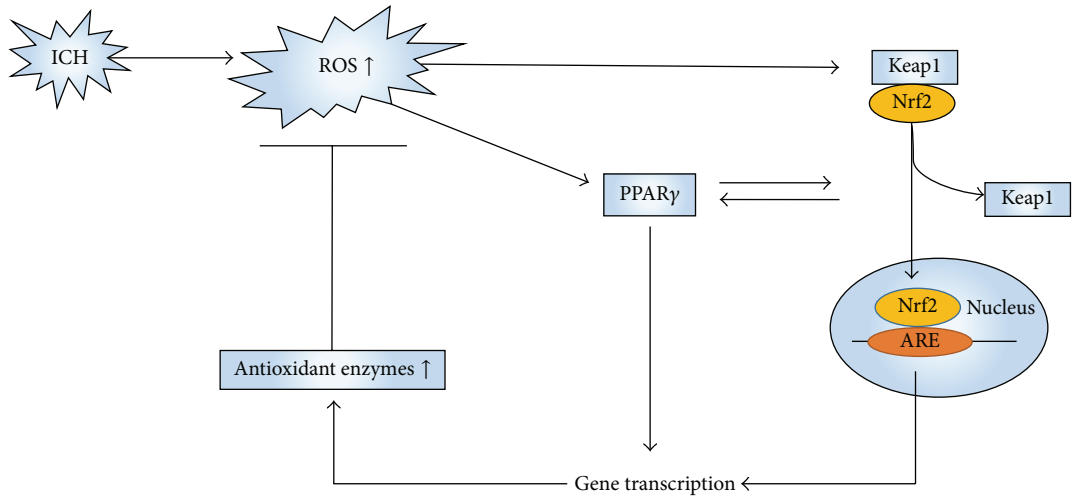

Figure 3: Oxidative stress can activate pathways involving Keap1 and Nrf2. Keap1 is a detector of ROS and a negative regulator of Nrf2. Under physiological conditions, Nrf2 is in a dormant state. When the brain is exposed to oxidative stress caused by ICH, Nrf2 will dissociate from Keap1, translocate to the nucleus, and activate antioxidant response element- (ARE-) dependent gene expression to neutralize ROS. Activation of PPARγ can lead to upregulation of the antioxidant enzymes catalase and superoxide dismutase (SOD). Nrf2: nuclear factor erythroid 2-related factor; Keap1: Kelch-like ECH-associated protein 1; ICH: intracerebral hemorrhage; ROS: reactive oxygen species; PPARγ: peroxisome proliferator-activated receptor gamma.

junctions (TJ), together with closely associated astrocytes, pericytes, and neurons, as well as the extracellular matrix [31]. Disruption of the BBB can cause brain edema, which is an important secondary injury after ICH. Edema is related to oxidative stress (Figure 2). Matrix metalloproteinases (MMPS) comprise a family of zinc-endopeptidases that can degrade TJ proteins and basal laminar proteins, increasing the permeability of the BBB. Many researchers have demonstrated that MMPs, particularly MMP-9, are upregulated after ICH, which is associated with oxidative stress [32]. Inhibition of oxidative stress via SOD1 overexpression also can decrease MMP-9 levels [7]. Therefore, ROS can trigger numerous molecular cascades that mediate the activation of MMPs, leading to BBB disruption.

5. Antioxidant System in Intracerebral Hemorrhage

To neutralize the rising ROS formed in ICH, the cellular antioxidant system is activated. The pathway involving Kelch-like ECH-associated protein 1 (Keap1) and nuclear factor erythroid 2-related factor 2 (Nrf2) is known as the major endogenous antioxidant system [33]. It was first discovered in studies of anticarcinogenic compounds, and a beneficial role of the pathway in the progression of ICH has been demonstrated [34] (Figure 3). Nrf2 knockout ($Nrf2^{-/-}$) mice have greater ROS production and DNA damage than wild type (WT) mice when subjected to 24 h of ICH [35]. Keap1 is a detector of ROS and a negative regulator of Nrf2. Under physiological conditions, Nrf2 is anchored within the cytoplasm by Keap1. When the brain is exposed to oxidative stress caused by ICH, Nrf2 will dissociate from Keap1, translocate to the nucleus, and activate antioxidant response element- (ARE-) dependent gene expression [35]. Shang et al. reported that, after blood infusion in a rat model, the expression of Keap1 began to decrease at 8 h, whereas Nrf2 began to show a significant increase at 2 h, with a peak at 24 h [36]. As a result of ARE's activation, different antioxidant enzymes, including superoxide dismutase (SOD), glutathione (GSH), hemeoxygenase-1 (HO-1), glutathione-S-transferase (GST), catalase, NADPH quinone oxidoreductase-1 (NQO1), and thioredoxin (TRX), are upregulated [35]. These enzymes can partly protect the cell from the oxidative stress caused by ICH. This pathway has also become a key target for therapies in ICH because of its pleiotropic antioxidant effects. Sulforaphane, an agent that can upregulate Nrf2, reduced injury caused by ICH in mice and rats via an Nrf-dependent mechanism [37]. Chang et al. found that, in the ICH mouse model induced by injecting collagenase, (−)-epicatechin, a brain-permeable flavanol, reduced the volume of the lesion and ameliorated neurologic deficits via activation of Nrf2-dependent and Nrf2-independent pathways [38]. Sukumari-Ramesh and Alleyne reported that tert-butylhydroquinone, a selective inducer of Nrf2, attenuated neurodegeneration and improved neurological outcomes after ICH [39]. Furthermore, peroxisome proliferator-activated receptor gamma (PPARγ) (Figure 3), which has pleiotropic effects regulating anti-inflammation and playing a role in glucose and lipid metabolism, appears to regulate the expression of Nrf2 [40, 41]. Activation of PPARγ also may directly lead to upregulation of antioxidant enzymes, such as catalase and superoxide dismutase [42, 43]. Therefore, PPARγ may be another endogenous antioxidant system.

6. Clinical Significance of ROS in ICH

6.1. Biomarkers in ICH. To understand the pathophysiology of oxidative stress in intracerebral hemorrhage and to identify

patient outcomes, we should take measures to evaluate oxidative stress. However, direct measurement of ROS in the brain is still difficult in humans. Therefore, several biological substances relevant to oxidative stress have been investigated as potential peripheral markers. In ischemic stroke, much research related to these biomarkers has demonstrated that biomarkers can be divided into two groups: (1) biomolecules damaged by ROS, including malondialdehyde (MDA); oxidized low-density lipoproteins (oxLDL); 8-isoprostaglandin-F-2 (8-iso-PGF2), a biomarker of lipid peroxidation; and 8-hydroxy-2-deoxy-guanosine (8-OHdG), a biomarker of DNA oxidation; (2) enzymes and molecules related to the antioxidant defence system, including superoxide dismutase (SOD), glutathione peroxidase (GPX), thioredoxin (Trx), and gamma-glutamyltransferase (GGT); vitamins A (retinol), C (ascorbic acid, AA), and E; and carotenoids [44]. However, during the pathological process of ICH, not all of the biomarkers mentioned in this passage can be detected. In a clinical study including 178 individuals (64 patients, 114 controls), ICH was significantly associated with an increased level of 8-OHdG, decreased GPX activity, and a decreased level of vitamin E, whereas MDA and vitamin A levels were not associated with ICH risk, and leukocyte 8-OhdG was better than traditional factors in predicting ICH outcome [45]. Uric acid (UA), an antioxidant molecule in human plasma, provides effective protection against oxidative stress in models of stroke. However, the relationship between uric acid and ICH prognosis is controversial; some authors have found that high levels of UA were correlated with a poor prognosis in ICH [46], whereas others did not conclude that uric acid levels were correlated with outcomes in ICH patients [47]. Vitamin C (ascorbic acid, AA) levels were also important indicators in ICH patients. AA levels were significantly inversely correlated with the severity of neurological impairment, as assessed by the Glasgow Coma Scale and the National Institutes of Health Stroke Scale, and with the major diameter of the lesion [48]. In conclusion, biomarkers of oxidative stress in ICH are similar to those of ischemic stroke, but there are differences between ICH and ischemic stroke, and assessment of multiple biomarkers may provide a better view of oxidative stress and outcomes in ICH patients.

6.2. Antioxidant Therapy in ICH. Therapeutic agents can be divided into two groups. One group includes agents that can prevent the formation of free radicals. Iron is a reactant in the formation of the hydroxyl radical, a highly reactive ROS, via the Fenton reaction, and iron chelating agents, such as clioquinol (CQ) and deferoxamine, reduced ICH-induced brain edema, neuronal death, and brain atrophy in a rat ICH model [49–51]. Apocynin, an inhibitor of NAD(P)H oxidase, can delay cerebral vasospasm in a rat subarachnoid hemorrhage model [52], and it also has protective effects on pups with intraventricular hemorrhage [53]. However, a neuroprotective effect was not observed in a rat ICH model [54]. Perhaps the amount of ROS derived from NAD(P)H oxidase is small compared with the total amount of ROS produced in ICH such that the effect of apocynin is limited. Sulfaphenazole (SPZ), which can inhibit superoxide production by cytochrome P450, is also a ROS scavenger. Hama et al. reported that systemic SPZ treatment reduces striatal dysfunction, elevated lipid peroxidation, and brain edema in a rat ICH model [55]. Thrombin can also initiate potentially harmful pathways, such as apoptosis in cultured neurons and astrocytes [56], and can activate Src kinase [57, 58], which may contribute to excitotoxicity and ROS release [59]. Therefore inhibition of thrombin may reduce injury induced by intracerebral hemorrhage. Mitochondria play an important role in ROS release in ICH, as mentioned in Section 3. Ma et al. reported that the mitochondrial ROS scavenger Mito-TEMPO can decrease the amount of the ROS in ICH and that TRO-19622, an inhibitor of the MPTP, can also reduce RIRR [24].

Agents that scavenge free radicals constitute the second group. These compounds include melatonin (5-methoxy-*N*-acetyl-tryptamine) and its metabolites, which are able to ameliorate early brain injury [60–62]. Melatonin has been shown to inhibit ROS related to red blood cell lysis and hemoglobin degradation. In addition, it can increase the expression of Nrf2-ARE pathway-related free radical scavengers, such as SOD and GSH, by activating the Nrf2-ARE pathway [63]. Hydrogen, the lightest element in the periodic table and the most abundant chemical substance in the universe, also can be used as an effective antioxidant therapy. Ohsawa et al. reported that inhaled hydrogen gas has antioxidant and antiapoptotic properties that protect the brain against ischemia-reperfusion (I/R) injury and stroke by selectively reducing the hydroxyl radical [64]. In the ICH model, inhalation of hydrogen gas can reduce brain edema acutely (24 h), but it is not effective over a longer time frame (72 h) [65]. *Momordica charantia* polysaccharide (MCP), obtained from *Momordica charantia*, can also be an antioxidant agent. Duan et al. also have demonstrated that MCP scavenged ROS in intracerebral hemorrhage damage, attenuating neuronal death in primary hippocampal neurons [66]. Pyrroloquinoline quinone (PQQ), which has been proven to exist in various fruits, vegetables, milk, and even mammalian tissues, can antagonize oxidative stress-induced cell damage [67–69]. Lu et al. have reported that PQQ reduced the production of reactive oxygen species, alleviated brain edema, and improved locomotor function after ICH [70]. Redox nanoparticles may become a new type of treatment for ROS. These agents can solve the problems of traditional agents, including nonspecific dispersion in normal issues, preferential renal clearance, poor permeability across the BBB, and rapid reduction to the corresponding hydroxylamine form. Chonpathompikunlert et al. reported that, in rats treated with redox polymer self-assembled nanoparticles (nitroxide radical-containing nanoparticles [RNPs]), significantly lower levels of superoxide anion and 8-OHdG (biomarkers of oxidative stress) were detected compared with rats in the control group. RNPs also ameliorated intracerebral hemorrhage-induced brain edema and neurological deficits [71].

There are many studies on antioxidant strategies in ICH, but there have been only a few drugs tested in clinical trials. Edaravone, a free radical scavenger, can significantly improve the outcome of ischemic stroke, as evaluated by the modified Rankin Scale, within 3 months and was introduced in Japan

for clinical use in June 2001 [72]. Its neuroprotective effect was also proven in preclinical trials [73–75]. However, in a meta-analysis of edaravone for acute intracerebral hemorrhage, which included 10 randomized controlled trials (RCTs) involving 768 participants, although edaravone treatment increased the rate of improvement of neurological impairment within the scheduled treatment, it is not clear that this translated to any longer-term benefit of clinical importance, and the quality of each trial was not high [76]. Therefore, the longer-term benefit of edaravone is still unclear. NXY059, a free radical-trapping neuroprotectant, is also a clinically tested antioxidant. However, in a randomized control trial including 607 patients, there were no differences in 3-month function, disability, or neurological deficit scores between the experimental group and the control group [77].

7. Conclusions

Reactive oxygen species are increasingly recognized as important players in the pathophysiology of secondary brain damage after ICH. Mitochondria play an important part in the production of ROS and are necessary to the processes of neuronal cell death and BBB injury. Much effort has been put into the development of antioxidants to neutralize oxidative stress. We have investigated many intracellular and mitochondrial targets, aiming to decrease ROS release or eliminate released ROS in ICH. However, we know that nearly all attempts failed. There may be two reasons for this. ROS in cells are not always harmful; they also play a role in molecular signal transduction. Therefore, treatment should be aimed at cytotoxic radicals. Additionally, we should pay more attention to the time window in which drugs are administered because it is not easy to supply patients with drugs in time. Moreover, the local and systemic influences of reactive oxygen species in ICH patients remain to be better characterized.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Jie Qu and Weixiang Chen contributed equally.

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Research Article

Metabolic Syndrome Augments the Risk of Early Neurological Deterioration in Acute Ischemic Stroke Patients Independent of Inflammatory Mediators: A Hospital-Based Prospective Study

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Background and Aims. Metabolic syndrome (MetS) has been associated with occurrence and prognosis of ischemic stroke. This study aimed to evaluate whether an association exists between MetS and early neurological deterioration (END) following acute ischemic stroke and the possible role inflammatory biomarkers play. *Methods and Results*. We conducted a prospective cohort investigation that involved 208 stroke patients within 48 hours from symptom onset. MetS was determined by the modified National Cholesterol Education Program/Adult Treatment Panel III criteria. END was defined as an increase of ⩾1 point in motor power or ⩾2 points in the total National Institutes of Health Stroke Scale (NIHSS) score within 7 days. Univariate logistic regression analysis showed that patients with MetS had a 125% increased risk of END (OR 2.25; 95% CI 1.71–4.86, $P = 0.005$). After adjustment for fibrinogen and high-sensitivity C-reactive protein, MetS remained significantly correlated to END (OR 2.20; 95% CI 1.10–4.04, $P = 0.026$) with a 77% elevated risk per additional MetS trait (OR 1.77; 95% CI 1.23–2.58, $P = 0.002$). *Conclusions*. This study demonstrated that MetS may be a potential predictor for END after ischemic stroke, which was independent of raised inflammatory mediators.

1. Introduction

In China, there are 1.5 to 2 million new stroke cases each year, and stroke has been ranked as the first leading cause of mortality and long-term disability [1]. Although most patients tend to improve in the first few days after stroke, a recognized fraction does not virtually recover but deteriorates, which has been termed as early neurological deterioration (END) [2]. Of note, END has been observed in approximately 5%–40% of patients with acute ischemic stroke [2–4]. And END is important for stroke prognosis because it may portend a higher risk of death and increased dependency in daily living [5–7]. Accordingly, it is important to detect and manage factors for END in order to improve stroke outcomes.

Metabolic syndrome (MetS), a constellation of interconnected vascular risk factors, includes insulin resistance/diabetes, elevated blood pressure, central obesity, and dyslipidemia [8]. Over the past two decades, the number of patients with MetS has enlarged strikingly in China [9]. There are a few studies reporting a positive relationship between MetS and unfavorable outcome after ischemic stroke [10, 11]. Nevertheless, whether MetS is a novel risk factor for acute stroke complication, such as END, has not been well addressed. On the other hand, high-sensitivity C-reactive protein (hsCRP) and fibrinogen, which are generally considered biomarkers of low-grade chronic systemic inflammation, have been implicated in the pathophysiology of MetS [12]. Meanwhile, it may be one of the molecular mechanisms

involved in ischemic stroke [13]. We further hypothesized that the association of MetS with an increased risk of END may be partially explained by higher levels of fibrinogen and hsCRP. We therefore performed this study to investigate the role of MetS and its components in the prediction of END in a hospital-based prospective study.

2. Experimental Methods

2.1. Study Populations. We prospectively screened patients in the Second Affiliated Hospital of Nanjing University of Chinese Medicine from Jan 2013 to Sep 2015. Patients with first-ever acute ischemic stroke aged 18 years or older and evaluated within 48 hours of symptom onset were included in the study. Patients with intravenous thrombolysis, history of brain surgery, tumor, presence of severe renal disease and hepatic disease, infectious disease, and early discharge were excluded. All participants consented to participation in the study in accordance with the research ethics attained through local ethics review committees.

2.2. Clinical Assessment. Data collection was performed using a standardized case report form. General information, previous medication history (including hypertension, diabetes mellitus, hyperlipidemia, atrial fibrillation, and coronary heart disease), the data of physical examination, clinical characteristics, laboratory data including fasting plasma glucose (FPG), hsCRP, fibrinogen, and imaging results were all recorded. Additional assessments consisting of carotid ultrasonography, transcranial Doppler, magnetic resonance angiography, and digital subtraction angiography were used to evaluate brain-supplying arteries. Cardiac diagnostic tests such as electrocardiography and transthoracic echocardiography were measured to identify cardioembolic stroke. Stroke subtype was classified according to TOAST (Trial of Org 10172 in Acute Stroke Treatment) criteria [14].

2.3. Treatment and Clinical Assessment Protocol. Once admitted in the stroke unit, guideline-based treatments, including monoantiplatelet, dual-antiplatelet, and anticoagulation, were performed in all participants immediately [15]. Oral statin supplementation is mandatory in cases without contradictions. Risk factors were managed according to guidelines during hospitalization.

Stroke severity was assessed by a certified neurologist using the National Institutes of Health Stroke Scale (NIHSS) at admission and continued 1–3 times a day for 7 days. In our study, END was defined as an increment of at least one point in motor power or total NIHSS score ⩾2 points deterioration within the first week after admission [16, 17].

2.4. Definition of MetS. According to the modified National Cholesterol Education Program/Adult Treatment Panel III (NCEP ATP III) criteria [18], MetS was recognized as the presence of at least three of the following risk components: (i) central obesity (waist circumference ⩾90 cm in men or ⩾80 cm in women); (ii) triglyceride (TG) ⩾1.70 mmol/L; (iii) high-density lipoprotein (HDL) cholesterol < 1.03 mmol/L (male) or < 1.30 mmol/L (female); (iv) elevated blood pressure: systolic blood pressure ⩾130 mmHg, diastolic blood pressure ⩾85 mmHg, or use for antihypertensive drugs; and (v) hyperglycemia: FPG ⩾5.6 mmol/L or need for antihyperglycemic medication.

2.5. Statistical Analysis. Statistical analysis was performed using SPSS software, version 17.0 (SPSS Inc., Chicago, IL). Continuous variables were presented as the means (SD) or medians (interquartile range, IQR) and analyzed with a t-test or Mann-Whitney U test. Categorical variables were expressed as n (%) and analyzed with a chi-square test or Fisher's exact test. One-way analysis of variance was used to evaluate the hsCRP and fibrinogen concentrations in different groups of individuals with 0 to 5 metabolic syndrome risk factors. Then, we performed multivariate logistic regression models to calculate odds ratios (OR) and 95% confidence intervals (CI) for the contribution of MetS and its components and the number of MetS components in the prediction of END. To determine whether inflammatory biomarkers may mediate the relationship between MetS and END, fibrinogen and hsCRP levels were added to the adjusted model. All tests were 2-tailed and statistical significance was established at P value of less than 0.05.

3. Results

Overall, 208 participants with an average age of 66.3 ± 9.2 (from 39 to 88 years old) were enrolled in this study. The mean time from symptom onset to initial evaluation was 24.6 ± 17.1 hours, and the median NIHSS score at admission was 3 points. More than 41% of the cohort met the criteria for MetS. Among patients with MetS, hypertension presented was the most prevalent MetS trait (96.5%), followed by obesity (80.4%), hyperglycemia (80.4%), hypertriglyceridemia (48.3%), and decreased HDL (18.4%). In total, 21.6%, 36.5%, 32.7%, and 9.1% patients had 0-1, 2, 3, and ⩾4 MetS traits, respectively.

MetS was more prevalent in females than males (51.5% versus 37.1%, $P = 0.049$). Notably, median hsCRP levels were 4.0 mg/L and 2.2 mg/L ($P = 0.039$) in patients with and without MetS. Among subjects with 0 to 5 MetS components, median hsCRP levels rose from 1.8 to 4.9 mg/L ($P = 0.019$). However, no association was found between fibrinogen levels and presence of MetS.

During the initial 7 days after admission, 49 patients were identified with END, which accounted for 23.6% [95% CI 20.7%–26.5%] of the cohort. Table 1 illustrated the baseline characteristics, inflammatory status, and MetS between the subgroups according to the presence or absence of END. Compared with patients without END, those with it were older (68.7±9.8 versus 65.6±8.9 years, $P = 0.040$), developing higher prevalence of diabetes mellitus (46.9% versus 25.8%, $P = 0.005$), MetS (59.2% versus 36.5%, $P = 0.005$), and increased number of MetS components ($P = 0.026$). We obtained the similar results when examining plasma inflammatory biomarkers. Patients with END had higher levels of leukocyte count (7.5 ± 1.5 versus 6.9 ± 1.810^9/L,

Table 1: Comparison of clinical characteristics between patients with and without END.

Characteristics	With END (n = 49)	Without END (n = 159)	P value
Age, year	68.7 ± 9.8	65.6 ± 8.9	0.040
Male (%)	32 (65.3)	108 (67.9)	0.733
Prehistory			
Smoking (%)	14 (28.6)	52 (32.7)	0.587
Drinking habits (%)	15 (30.6)	43 (27.0)	0.626
Hypertension (%)	31 (63.3)	120 (75.5)	0.101
Diabetes (%)	23 (46.9)	41 (25.8)	0.005
Hyperlipidemia (%)	10 (20.4)	28 (17.6)	0.658
Atrial fibrillation (%)	6 (12.2)	12 (7.5)	0.307
Coronary heart disease (%)	6 (12.2)	16 (10.1)	0.664
Previous antiplatelet (%)	4 (8.2)	17 (10.7)	0.607
Previous stain (%)	3 (6.1)	15 (9.4)	0.471
SBP, mmHg	150.2 ± 17.1	148.7 ± 17.8	0.603
DBP, mmHg	85.0 ± 10.6	85.1 ± 11.6	0.971
BMI, kg/m^2	25.5 ± 1.6	25.8 ± 1.7	0.242
NIHSS, score	3 (2, 5)	3 (2, 4)	0.193
Time from onset to admission, h	24 (12, 24)	24 (8, 24)	0.970
Length of stay, day	18 (15.5, 21.5)	13 (11, 14)	0.001
MetS (%)	29 (59.2)	58 (36.5)	0.005
Number of metabolic factors			0.026
0-1	5 (10.2)	40 (25.2)	
2	15 (30.6)	61 (38.4)	
3	22 (44.8)	46 (28.9)	
4-5	7 (14.3)	12 (7.5)	
Stroke subtype (TOAST)			0.864
LAA	28 (57.1)	90 (56.6)	
CE	5 (10.2)	11 (6.9)	
SVO	14 (28.6)	52 (32.7)	
Others	2 (4.1)	6 (3.8)	
Laboratory data			
Leukocyte count, 10^9/L	7.5 ± 1.5	6.9 ± 1.8	0.038
Platelet count, 10^9/L	186 (156, 228)	190 (145, 210)	0.817
Fibrinogen, mg/dL	307.8 ± 98.7	286.6 ± 66.8	0.088
hsCRP, mg/L	6.0 (2.0, 11.5)	2.0 (0.9, 5.0)	0.001
Homocysteine, umol/L	14.4 (12.0, 21.7)	11.1 (13.0, 18.0)	0.049
Fasting plasma glucose, mmol/L	8.0 (5.9, 9.9)	5.4 (5.0, 6.1)	0.001
TC, mmol/L	4.8 ± 1.3	4.5 ± 1.0	0.194
TG, mmol/L	1.3 (1.0, 2.2)	1.3 (1.0, 1.7)	0.307
HDL, mmol/L	1.4 (1.1, 1.6)	1.3 (1.1, 1.5)	0.192
LDL, mmol/L	2.3 (1.7, 2.9)	2.3 (1.9, 2.9)	0.750

MetS, metabolic syndrome; END, early neurological deterioration; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; LAA, large artery atherosclerosis; SVO, small vessel occlusion; CE, cardioembolism.

P = 0.038), hsCRP (6.0 versus 2.0 mg/L, P = 0.001), and homocysteine (14.4 versus 11.1 umol/L, P = 0.049), with longer hospital stay (18.0 versus 13.0 day, P = 0.001).

Univariate logistic regression analysis revealed that MetS was positively correlated to increasing risk of END in ischemic stroke patients (OR 2.25; 95% CI 1.71–4.86, P = 0.005). Only one MetS component, hyperglycemia, was associated with greater END risk (OR 5.83; 95% CI 2.81–12.08, P < 0.001). Findings were similar when measured according to the number of MetS traits (Figure 1). After adjusting for age and sex, hyperglycemia (OR 6.33; 95% CI 2.99–13.39, P < 0.001) and MetS (OR 3.08; 95% CI 1.54–6.16, P = 0.004) were

Table 2: Logistic regression analysis for the association of MetS and its components with END in ischemic stroke patients.

	Univariate analysis OR (95% CI)	Model 1 OR (95% CI)	Model 2 OR (95% CI)
Central obesity	0.98 (0.51–1.88)	1.04 (0.52–2.10)	0.84 (0.42–1.68)
Hyperglycemia	5.83 (2.81–12.08)[b]	6.33 (2.99–13.39)[b]	5.259 (2.49–11.12)[b]
Elevated blood pressure	0.79 (0.34–1.84)	0.76 (0.32–1.78)	0.86 (0.35–2.08)
Decreased HDL	0.92 (0.35–2.42)	1.04 (0.38–2.86)	0.92 (0.31–2.75)
Hypertriglyceridemia	1.69 (0.85–3.38)	1.98 (0.96–4.06)	1.83 (0.88–3.82)
MetS	2.25 (1.71–4.86)[a]	3.08 (1.54–6.16)[a]	2.20 (1.10–4.04)[a]

MetS, metabolic syndrome; END, early neurological deterioration; HDL, high-density lipoprotein; OR, odds ratios; CI, confidence interval; [a]$P < 0.05$; [b]$P < 0.001$; Model 1 adjusted for age and gender; Model 2 adjusted for levels of fibrinogen and hsCRP.

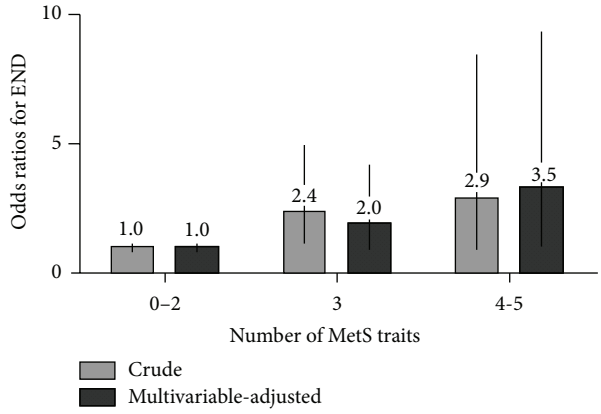

Figure 1: Odds ratios for END according to the number of MetS traits. MetS, metabolic syndrome; END, early neurological deterioration. Data are crude (light bars) and multivariable-adjusted (dark bars) odds ratios. Multivariable model was adjusted for levels of fibrinogen and high-sensitivity C-reactive protein. Error bars represent 95 percent confidence intervals.

related with END (Model 1, Table 2). This trend remained significant after controlling for levels of fibrinogen and hsCRP (Model 2, Table 2).

4. Discussion

Our prospective study found that ischemic stroke patients with MetS were at increasing risk of developing END. We also showed that the risk of END was positively associated with the accumulation of MetS components. Risk relationships were not appreciably attenuated after adjustment for levels of fibrinogen and hsCRP, suggesting that the excess risk with MetS may not be mediated by heightened inflammation.

In contrast to later neurological deterioration which usually results from systemic complications, END is more likely to be related to biochemical abnormality such as hyperglycemia and inflammation [19]. As similar to previous studies [20, 21], our cohort demonstrated 5.8-fold increased odds ratios of END (OR 5.83; 95% CI 2.81–12.08, $P < 0.001$) among those with hyperglycemia. Possible mechanisms of hyperglycemia-associated neurological deterioration could be the fact that it induces endothelial damage, intracellular acidosis, and blood-brain barrier disruption [22]. Also, several observational studies have suggested that inflammation may play a critical role in END [23, 24]. Vila et al. [23] found that interleukin-6 in plasma (21.5 pg/mL; OR 37.7, 95% CI 11.9–118.8) and cerebrospinal fluid (>6.3 pg/mL; OR 13.1, 95% CI 2.2–77.3) were significant factors for early clinical worsening in all ischemic stroke subtypes, independent of initial size and topography. Castellanos et al. [24] performed a secondary analysis of 113 consecutive patients with lacunar infarction and reported that high concentrations of interleukin-6, tumor necrosis factor-α, and intercellular adhesion molecule-1 in blood were associated with END and Barthel Index < 85. Moreover, plasma interleukin-10, a well-known anti-inflammatory cytokine, was found to be protective for END on multivariate analysis (OR 0.3, 95% CI 0.1–0.9) [25]. Nevertheless, the precise signals underlying END mediated by inflammation are uncertain but may involve neurotoxicity, particularly in conditions of local hypoxia [26]. Thus, identifying metabolic markers for END will conduce to detection of its potential mechanisms and target therapeutic interventions for prevention.

MetS is a growing public health problem worldwide. The prevalence of MetS has reached 58% in elderly Chinese population and it is projected to increase considerably [27]. Evidently MetS has been reported to augment the risk of stroke (relative risk [RR]: 2.27; 95% CI: 1.80 to 2.85), cardiovascular disease (RR: 2.35; 95% CI: 2.02 to 2.73), and all-cause mortality (RR: 1.58; 95% CI: 1.39 to 1.78) [28]. Also, in a study population of 691 subjects with acute ischemic stroke [11], MetS was independently correlated to a higher modified Rankin Scale score at discharge (OR 1.57; 95% CI 1.13–2.19), which was prominent with more MetS traits after being controlled for other risk factors ($P = 0.030$). To our knowledge, the impact of MetS on acute stroke complications has not been evaluated. Our present study implied that MetS increased the incidence of END approximately 2.3-fold (OR 2.25; 95% CI 1.71–4.86, $P = 0.005$). As shown in previous studies [20, 21, 29], metabolic abnormalities that integrate MetS have been in a close relation with aggravation of acute ischemic stroke. MetS-related alterations comprise

impairments in endogenous fibrinolytic capacity, endothelial dysfunction, and a proinflammatory state, all of which may contribute to neurological deterioration [30].

It is worthwhile to mention that, in accordance with prior studies [31–33], our data also confirmed that levels of plasma hsCRP (OR 1.05; 95% CI 1.01–1.09, $P = 0.015$), a marker of inflammation, were strongly correlated to MetS. We also found an increase in plasma levels of hsCRP (from 1.8 mg/L to 4.9 mg/L) per additional MetS component. Furthermore, in other prospective studies, higher levels of circulating hsCRP [34], leukocyte count [35], and interleukin-6 [23, 24] have been found to increase the risk of early neurological worsening. Therefore, one potential explanation for findings reported here is that elevated levels of inflammatory biomarkers may mediate the association of MetS with END, whereas when fibrinogen and hsCRP levels were added to the adjusted model, MetS remained a significant associated factor (OR 2.20; 95% CI 1.10–4.04, $P = 0.026$) for END in our study. This is a novel finding which suggested that the relationship between MetS and END may not be mediated by circulating levels of hsCRP and fibrinogen. It is possible that acute cerebral inflammatory responses caused by local cytokines may contribute to END [36]. Other potentially biological mediators may include adiponectin decrease [37], which exerts inflammatory function and exacerbates insulin resistance and gamma-glutamyl transpeptidase increment that disrupts intracellular homeostasis of oxidative stress [38, 39]. Herein, further study is needed to identify the biological pathway by which MetS promotes END.

Several limitations should be stressed in the present study. Firstly, the study was conducted in one center with small sample size, which may generate sampling bias. Secondly, the conception of END varies among different studies [2, 3, 5–7]. However, the definition in our study has been widely recommended by researchers, because worse outcomes have been demonstrated in patients under this scoring [40]. Finally, data were observational. Relationships reported cannot be proved as causality. These issues should be addressed in future multicenter studies.

In summary, the occurrence of END among patients with acute ischemic stroke in our study was 23.6%, which was consistent with previous data ranging from 5% to 40%. MetS may be predictive of END, especially the higher levels of plasma glucose, while this association may not be mediated by systemic inflammation. Further studies with large sample size are needed to investigate these associations comprehensively. Pathophysiological mechanisms and therapeutic considerations also remain to be determined.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Lower Serum Caveolin-1 Is Associated with Cerebral Microbleeds in Patients with Acute Ischemic Stroke

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Caveolin-1 (Cav-1) plays pivotal roles in the endothelial damage following stroke. The present study aimed to investigate whether serum Cav-1 level is associated with the presence of cerebral small vessel disease (cSVD) in patients with acute ischemic stroke. To this end, 156 patients were consecutively enrolled. Cranial magnetic resonance imaging was analyzed to determine the surrogates of cSVD, including cerebral microbleeds (CMBs), silent lacunar infarcts (SLIs), and white matter hyperintensities (WMHs). After adjusting for potential confounders, patients with low Cav-1 level had a higher risk of CMBs than patients with high Cav-1 level (OR: 4.05, 95% CI: 1.77–9.30). However, there was no relationship between Cav-1 and the presence of SLIs or WMHs. When CMBs were stratified by location and number, a similar association was found in patients with deep or infratentorial CMBs (OR: 4.04, 95% CI: 1.59–10.25) and with multiple CMBs (OR: 3.18, 95% CI: 1.16–8.72). These results suggest lower serum Cav-1 levels may be associated with CMBs, especially those that are multiple and located in deep brain or infratentorial structures, in patients with acute ischemic stroke. Cav-1 may be involved in the pathophysiology of CMBs, and may act as a potential target for treating cSVD.

1. Introduction

Caveolae are small flask-shaped invaginations of the cell plasma membrane and are particularly abundant in endothelial cells. They participate in many intra- and intercellular activities, such as endocytosis, vesicular trafficking, and signal transduction [1, 2]. As a key structural protein of caveolae domains, caveolin-1 (Cav-1) participates in regulating blood-brain barrier (BBB) permeability, oxidative stress, and counteracting neuroinflammatory process [3, 4]. In laboratory stroke investigations, Cav-1 levels have been associated with neuronal apoptosis, BBB disruption, infarction enlargement, and functional deterioration [5, 6], yet there has been no clinical research to confirm these relationships to date.

Cerebral small vessel disease (cSVD) is mainly characterized by cerebral microbleeds (CMBs), silent lacunar infarcts (SLIs), and white matter hyperintensities (WMHs) in magnetic resonance imaging (MRI) [7]. These trinity imaging surrogates have been associated with increased risk of subsequent cerebrovascular events and unfavorable functional outcomes in ischemic stroke survivors [8–12]. Although potential unknown mechanisms are thought to participate in the development of cSVD, previous studies demonstrated that inflammatory cascade activated by endothelial lesion and BBB damage is involved in the initiation and progression of cSVD [7, 13]. Considering a salient role of Cav-1 in these pathophysiologic processes, we hypothesized that Cav-1 levels might be related to the presence of cSVD. Therefore, in this study, we investigated the association between serum Cav-1 levels and cSVD detected and measured with MRI in a cohort of patients with ischemic stroke.

2. Subjects and Methods

2.1. Study Subjects. From January 2013 to December 2014, patients with ischemic stroke registered in Nanjing Stroke

Registry Program (NSRP) were prospectively enrolled. NSRP has been described previously in detail [14]. Patients were enrolled in this study if they are (1) with first-ever ischemic stroke within 24 hours of symptom onset and (2) aged 18 years or older. We excluded patients if they are (1) with traumatic brain injury or brain surgery, (2) with chronic kidney diseases, (3) with malignant tumor, and (4) currently with infectious disease. All patients provided informed consent, and the study was approved by the ethics committee of Jinling Hospital.

According to the inclusion criteria, there were 172 patients with first-ever ischemic stroke within 24 hours of symptom onset and 18 years or older. The ischemic stroke was confirmed by professional neurologist with acute stroke syndrome and subsequent imaging examination. During this period, 7 patients refused to participate. Additionally, 2 patients with traumatic brain injury or brain surgery, 2 with chronic kidney disease, 2 with malignant tumor, and 3 with infectious disease were excluded. Accordingly, a total of 156 patients were included in final analysis.

2.2. MRI Protocols. Cranial MRI imaging was performed with a 3.0 T Trio MRI scanner (Siemens, Erlangen, Germany) with a 12-channel head coil. The imaging protocol consisted of axial T1-weighted, T2-weighted, fluid-attenuated inversion recovery (FLAIR), diffusion-weighted imaging (DWI), and susceptibility-weighted imaging (SWI) sequences. T1-weighted, T2-weighted, FLAIR, and DWI sequences were performed according to previously standardized procedures and parameters: T1-weighted: repetition time (TR), 350 ms; echo time (TE), 2.46 ms; T2-weighted: TR, 4000 ms; TE, 98 ms; T2-FLAIR: TR, 7000 ms; TE, 87 ms; DWI: TR, 3000 ms; TE, 91 ms. SWI was performed with the following parameters: matrix size, $512 \times 254 \times 72$; field of view (FOV), $230 \times 115 \times 144\ mm^3$; TR, 56 ms; TE, 25 ms; flip angle, 20°. The detail has been described in our previous study [15].

2.3. Cranial MRI Surrogates of cSVD. The MRI surrogates of cSVD of interest were CMBs, SLIs, and WMHs. Imaging results from all included patients ($n = 156$) were assessed by 2 raters who were blind to the clinical information. Disagreements were resolved by consultation with a third reviewer. CMBs were defined as small round hypointensity areas (2–10 mm in diameter) on SWI sequence [16, 17] and were categorized according to their locations as lobar (cortical gray and subcortical or periventricular white matter), deep (deep gray matter: basal ganglia and thalamus, and the white matter of the corpus callosum, internal, eternal, and extreme capsule), and infratentorial (brainstem and cerebellum). Furthermore, patients with CMBs were dichotomized according to the presence or absence of microbleeds in deep or infratentorial locations. Those with microbleeds in deep or infratentorial locations were defined as "deep or infratentorial microbleeds" and those without were defined as "strictly lobar microbleeds" [18]. CMBs were further categorized according to their numbers as absent, single (1 CMB), and multiple (≥2 CMBs). CMBs mimics, such as calcifications, iron deposits, and flow voids in pial blood vessel were carefully excluded [16, 17]. Interrater reliability for the presence of CMBs was 0.85.

SLIs were defined as focal lesions (3–15 mm in diameter), accompanied by hypointensity on T1 image, corresponding hyperintensity on T2 image, and hypointensity with perifocal high signal on T2 FLAIR image [19]. WMHs were defined as hyperintensity surrounding the ventricles and in the deep white matter on FLARI images, classified by a modified Fazekas rating scale. Periventricular hyperintensity was graded as 0 = absence, 1 = "caps" or pencil-thin lining, 2 = smooth "halo," or 3 = irregular extending into the deep white matter. Deep white matter hyperintensity was rated as 0 = absence, 1 = punctate foci, 2 = beginning confluence of foci, or 3 = large confluent areas [20]. The presence of WMHs was defined when grade is ≥1 of any location. Interrater reliability for the presence of SLIs and WMHs was 0.78 and 0.81, respectively.

2.4. Cerebrovascular Risk Factors. Baseline characteristics of patients were collected at the time of admission. Hypertension was defined as systolic blood pressure (SBP) ≥ 140 mmHg, diastolic blood pressure (DBP) ≥ 90 mmHg, or use of antihypertensive medications. Diabetes mellitus was diagnosed if either the fasting glucose level was ≥126 mg/dL or the participants were currently being treated with antidiabetic agents. Hyperlipidemia was defined as an elevated level of triglycerides (≥150 mg/dL), total cholesterol (≥220 mg/dL), or low-density lipoprotein cholesterol (≥140 mg/dL) or having received lipid-lowering drugs. Coronary heart disease, atrial fibrillation, and myocardial infarction were all recognized as heart disease. Body mass index (BMI) was calculated as weight (kg)/height (m^2). Smoking was defined as currently smoking or having quit for ≤2 years. Drinking was defined as current alcohol drinking ≥20 g/d.

2.5. Serum Cav-1 Measurement. Morning blood samples for measurements of glucose, lipid, fibrinogen, and Cav-1 levels were obtained after an overnight fast within 48 hours of symptom onset. Blood was centrifuged at 1500 g for 10 minutes within 30 minutes of collection, and the serum was stored at -80°C. Cav-1 levels were measured by commercially available enzyme-linked immunosorbent assay kit (Uscn Life Science, Wuhan, China) according to the manufacturer's instructions. The intra-assay coefficient was <10%.

2.6. Statistical Analysis. Continuous variables were expressed as mean ± SD or median (interquartile range) and compared with Student's t-test or Mann-Whitney U test. Categorical variables were expressed as percentages and compared using Chi-square or Fisher's exact test. Logistic regression was used to evaluate the relationship between Cav-1 and cSVD by calculating adjusted odds ratios (OR) and 95% confidence intervals (CI). Cav-1 levels were divided into dichotomy with cut points of 5.25 ng/mL. Variables at a level of $P < 0.1$ in univariable comparison and those being reported previously as potential confounders were adjusted in multivariable logistic regression, which were first adjusted for age and sex (Model 1) and then for all potential confounders (Model 2). A two-tailed α value of 0.05 was deemed statistically significant. All data analyses were performed using SPSS 19.0.

Table 1: Baseline characteristics of patients according to the presence of cSVD.

Variables	All patients $n = 156$	CMBs (+) $n = 57$	SLIs (+) $n = 82$	WMHs (+) $n = 95$
Age, y	63.2 ± 9.1	65.1 ± 8.4*	65.0 ± 8.7**	64.7 ± 9.0*
Male, %	107 (68.6)	44 (77.2)	60 (73.2)	62 (65.3)
Hypertension, %	104 (66.7)	44 (77.2)*	56 (68.3)	70 (73.7)*
Diabetes mellitus, %	37 (23.7)	16 (28.1)	22 (26.8)	23 (24.2)
Dyslipidemia, %	52 (33.3)	15 (26.3)	25 (30.5)	30 (31.6)
Heart disease, %	22 (14.1)	8 (14.0)	14 (17.1)	13 (13.7)
BMI, kg/m^2	25.1 (23.9–26.0)	24.9 (24.0–25.9)	25.3 (23.9–26.1)	24.9 (23.9–25.8)
Smoking, %	54 (34.6)	19 (33.3)	28 (34.1)	35 (36.8)
Alcohol intake, %	59 (37.8)	22 (38.6)	33 (40.2)	38 (40.0)
SBP, mmHg	140 (130–145)	140 (131–149)*	140 (130–148)*	140 (130–148)*
DBP, mmHg	85 (80–90)	90 (80–95)	85 (80–90)	86 (80–90)
Antithrombotics use, %	58 (37.2)	18 (31.6)	29 (35.4)	37 (38.9)
Onset-to-MRI time, day	2 (1-2)	2 (1-2)	2 (1-2)	2 (1-2)
Onset-to-blood drawing time, day	2 (1-2)	2 (1-2)	2 (1-2)	2 (1-2)
NIHSS	3 (2–5)	3 (2–4.5)	3 (2–5)	3 (2–6)
Fasting glucose, mg/dL	91.8 (85.1–105.6)	92.2 (84.6–116.3)	91.8 (84.6–104.8)	93.6 (86.4–108.0)
Triglycerides, mg/dL	119.0 (89.4–160.2)	113.3 (87.2–149.1)	107.1 (87.4–151.8)	119.5 (87.6–159.3)
HDL, mg/dL	40.8 ± 11.4	41.8 ± 12.9	41.1 ± 11.0	41.8 ± 11.7
LDL, mg/dL	95.0 ± 27.8	93.8 ± 22.2	94.1 ± 26.9	95.7 ± 24.3
Total cholesterol, mg/dL	157.2 ± 36.1	155.9 ± 30.5	155.6 ± 36.3	157.6 ± 34.9
Fibrinogen, mg/dL	296.1 ± 59.4	310.9 ± 61.1*	301.2 ± 65.3	295.8 ± 58.1
Cav-1, ng/mL	5.62 ± 2.63	4.74 ± 2.26**	5.71 ± 2.77	5.65 ± 2.58

CMBs, cerebral microbleeds; SLIs, silent lacunar infarcts; WMHs, white matter hyperintensities; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; NIHSS, National Institutes of Health Stroke Scale; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Cav-1, caveolin-1.
* $P < 0.05$; ** $P < 0.01$ compared with corresponding negative (−) group.

3. Results

3.1. Baseline Characteristics. A total of 156 patients (68.6%, male; mean age, 63.2 ± 9.1 years) were enrolled in the study. The baseline characteristics of the patients were presented in Table 1. Overall, 104 (66.7%) patients had hypertension, 37 (23.7%) patients had diabetes mellitus, 52 (33.3%) patients had hyperlipidemia, and 22 (14.1%) patients had heart disease. The mean ± SD (range) Cav-1 level of these patients was 5.62 ± 2.63 (1.67–12.32) ng/mL.

Based on MRI results, there were 57 (36.5%) patients with CMBs, 82 (52.6%) patients with SLIs, and 95 (60.9%) patients with WMHs. Univariable comparison showed that patients with CMBs were older than patients without (65.1 ± 8.4 versus 62.1 ± 9.4, $P = 0.046$). Patients with CMBs had higher incidence of hypertension (77.2% versus 60.6%, $P = 0.034$) and higher systolic blood pressure (140 versus 135 mmHg, $P = 0.013$) at admission. The levels of blood fibrinogen were higher (310.9±61.1 versus 287.6±57.1 mg/dL, $P = 0.018$) in patients with CMBs than in patients without. Patients with CMBs had lower serum Cav-1 levels (4.74 ± 2.26 versus 6.12 ± 2.71 ng/mL, $P = 0.001$) than patients without. For ischemic cSVD, patients with SLIs were older than patients without (65.0 ± 8.7 versus 61.2 ± 9.2, $P = 0.009$). Systolic blood pressure at admission was higher in patients with SLIs (140 versus 137 mmHg, $P = 0.029$) than in patients without. Similarly, patients with WMHs were older than patients without (64.7 ± 9.0 versus 60.9 ± 8.9, $P = 0.011$). And patients with WMHs had higher prevalence of hypertension (73.7% versus 55.7%, $P = 0.020$) and higher systolic blood pressure at admission (140 versus 134 mmHg, $P = 0.015$) than patients without. However, there were no significant differences concerning the levels of serum Cav-1 between patients with SLIs and patients without (5.71 ± 2.77 versus 5.51 ± 2.49 ng/mL, $P = 0.634$) or between patients with WMHs and patients without (5.65 ± 2.58 versus 5.58 ± 2.74 ng/mL, $P = 0.875$, Figure 1).

3.2. Relationship between Cav-1 and cSVD. After adjusting for age and sex (model 1), presence of CMBs in patients with low Cav-1 level (≤5.25 ng/mL) was higher (OR = 3.69, 95% CI 1.78–7.64, $P = 0.00044$) than that in patients with high Cav-1 level (>5.25 ng/mL). After adjusting for more confounders (model 2), presence of CMBs was still higher in patients with low Cav-1 level (OR = 4.05, 95% CI 1.77–9.30, $P = 0.001$) than that in patients with high Cav-1 level. However, there were no significant differences between Cav-1 and SLIs (OR = 1.61, 95% CI 0.77–3.36, $P = 0.209$, Model 2) or WMHs (OR = 0.77, 95% CI 0.36–1.67, $P = 0.510$, Model 2), respectively (Table 2).

3.3. Relationship between Cav-1 and CMBs Subgroups. Of the 57 patients with CMBs, 22 (38.6%) were categorized as being with single CMBs and 35 (61.4%) as being with multiple CMBs. CMBs were more frequently observed in deep brain or infratentorial structures (42/57, 73.7%) than in lobar areas (15/57, 26.3%). When CMBs were categorized according to their locations, patients with CMBs in deep brain or

Table 2: Logistic regression analysis for association between Cav-1 and cSVD.

Cav-1	CMBs				SLIs				WMHs			
	Model 1	*P*	Model 2	*P*	Model 1	*P*	Model 2	*P*	Model 1	*P*	Model 2	*P*
≤5.25 ng/mL	3.69 (1.78–7.64)	0.00044	4.05 (1.77–9.30)	0.001	1.30 (0.68–2.50)	0.431	1.61 (0.77–3.36)	0.209	0.88 (0.45–1.70)	0.696	0.77 (0.36–1.67)	0.510
>5.25 ng/mL	Reference		Reference		Reference		Reference		Reference		Reference	

Model 1, adjusted for age and sex; Model 2, adjusted for age, sex, hypertension, diabetes mellitus, dyslipidemia, heart disease, BMI, smoking, alcohol intake, SBP, DBP, antithrombotics use, onset-to-MRI time, onset-to-blood drawing time, NIHSS, fasting glucose, triglycerides, HDL, LDL, total cholesterol, and fibrinogen.
CMBs, cerebral microbleeds; SLIs, silent lacunar infarcts; WMHs, white matter hyperintensities.

Table 3: Logistic regression analysis for association between Cav-1 and CMBs subgroups.

Cav-1	CMBs location							
	Deep or infratentorial CMBs (n = 42)				Strictly lobar CMBs (n = 15)			
	Model 1	P	Model 2	P	Model 1	P	Model 2	P
≤5.25 ng/mL	3.54 (1.58–7.92)	0.002	4.04 (1.59–10.25)	0.003	1.85 (0.60–5.69)	0.286	1.55 (0.43–5.60)	0.501
>5.25 ng/mL	Reference		Reference		Reference		Reference	
Cav-1	CMBs number							
	Multiple CMBs (n = 35)				Single CMBs (n = 22)			
	Model 1	P	Model 2	P	Model 1	P	Model 2	P
≤5.25 ng/mL	2.70 (1.18–6.17)	0.018	3.18 (1.16–8.72)	0.025	2.72 (0.99–7.45)	0.052	3.03 (0.95–9.72)	0.062
>5.25 ng/mL	Reference		Reference		Reference		Reference	

Model 1, adjusted for age and sex; Model 2, adjusted for age, sex, hypertension, diabetes mellitus, dyslipidemia, heart disease, BMI, smoking, alcohol intake, SBP, DBP, antithrombotics use, onset-to-MRI time, onset-to-blood drawing time, NIHSS, fasting glucose, triglycerides, HDL, LDL, total cholesterol, and fibrinogen. CMBs, cerebral microbleeds.

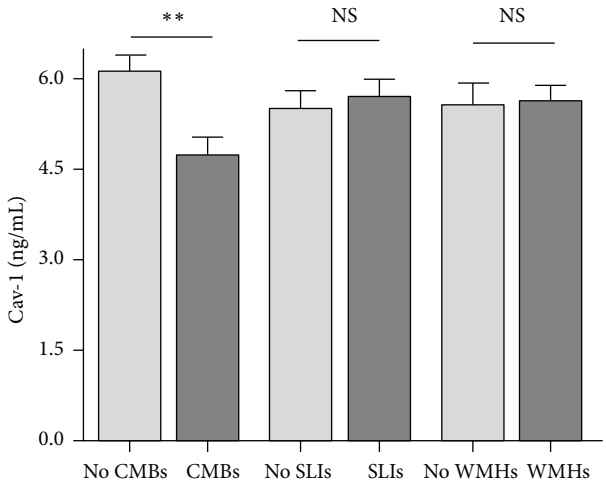

Figure 1: Mean ± SEM of Cav-1 levels are shown according to the presence of cSVD. $^{**}P < 0.01$ compared with corresponding negative group. CMBs indicate cerebral microbleeds. SLIs indicate silent lacunar infarcts. WMHs indicate white matter hyperintensities. Cav-1 indicates caveolin-1.

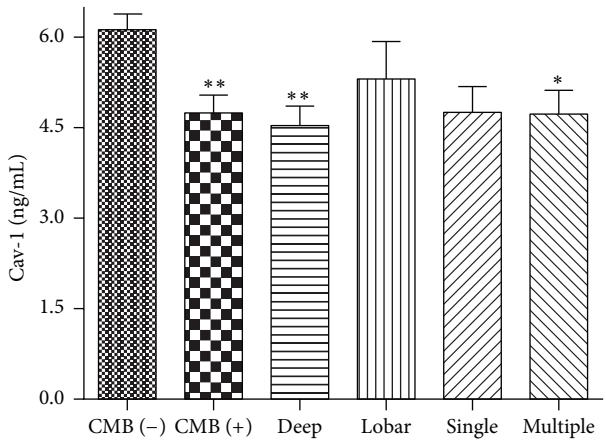

Figure 2: Mean ± SEM of Cav-1 levels are shown according to the presence, location, and number of CMBs. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with CMBs (−) group. CMBs indicate cerebral microbleeds. Cav-1 indicates caveolin-1.

infratentorial structures had significantly lower levels of Cav-1 than patients without CMBs (4.53 ± 2.22 versus 6.12 ± 2.71 ng/mL, $P = 0.003$). However, there was no significant difference concerning Cav-l levels between patients with lobar CMBs and patients without CMBs (5.32 ± 2.37 versus 6.12 ± 2.71 ng/mL, $P = 0.764$). When CMBs were categorized according to their numbers, patients with multiple CMBs had lower levels of Cav-1 than patients without CMBs (4.72 ± 2.43 versus 6.12 ± 2.71 ng/mL, $P = 0.019$, Figure 2).

Logistic regression analysis showed that the presence of deep or infratentorial CMBs was higher in patients with low Cav-1 levels than that in patients with high Cav-1 level (OR = 3.54, 95% CI 1.58–7.92, $P = 0.002$, Model 1). After adjusting for more confounders, the association still existed (OR = 4.04, 95% CI 1.59–10.25, $P = 0.003$, Model 2). Similar association was found between Cav-1 and the presence of multiple CMBs (OR = 3.18, 95% CI 1.16–8.72, $P = 0.025$, Model 2). However, no association was found between Cav-1 and strictly lobar CMBs (OR = 1.55, 95% CI 0.43–5.60, $P = 0.501$, Model 2) or single CMBs (OR = 3.03, 95% CI 0.95–9.72, $P = 0.062$, Model 2), respectively (Table 3).

4. Discussion

This study found that lower serum Cav-1 level is associated with the presence of CMBs in acute ischemic stroke. After adjusting for potential confounders, patients with low Cav-1 level had a 3-fold increased risk of CMBs compared with patients with high Cav-1 level. When CMBs were categorized according to number and location, the low serum Cav-1 levels were independently associated with multiple CMBs and deep or infratentorial CMBs.

Growing evidence has suggested that Cav-1 is involved in the regulation of lipoprotein transcytosis across endothelial cells and in the regulation of vascular inflammation and mitochondrial oxidative metabolism. Cav-1 knockout (KO) ischemic stroke models exhibited increased BBB permeability, redox imbalance, and amplified proinflammatory cytokines [3, 21]. When Cav-1 was downregulated, there

was an increase of matrix metalloproteinases- (MMP-) 2/9 activity, which can hydrolyze BBB extracellular matrix and tight junction (TJ) proteins and subsequently leads to the BBB opening [22]. As such, decreased Cav-1 expression can increase the activation of endothelial nitric oxide synthase (eNOS), which increases NO production in endothelial cells and leads to remarkably endothelial and microvascular hyperpermeability [23, 24]. On the other hand, downregulation of Cav-1 was reported to have amplified proinflammatory cytokines, including IL-1β, IL-2, IL-6, and IL-9 [3, 19]. Deficiency of Cav-1 presented altered redox homeostasis and promoted a significant increase of oxidative stress in endothelial cell, which possibly reflects a role of Cav-1 in mitochondrial function [25]. These molecular mechanisms are also supposed to be associated with the occurrence of cSVD. As found in this clinical study, lower serum Cav-1 levels were associated with the presence of CMBs.

CMBs, as a novel image surrogate of cSVD, have been proved to be associated with endothelial dysfunction, BBB leakage, inflammation activation, and oxidative stress [7, 13, 19, 26]. Histopathological studies demonstrate that the spatial distribution of CMBs may reflect specific underlying vascular pathological changes, in particular cerebral amyloid angiopathy (CAA) and hypertensive vasculopathy [17]. These two disorders are characterized by different patterns of microbleeds distribution: hypertensive vasculopathy is usually associated with CMBs in the basal ganglia, thalamus, brainstem, and cerebellum [27], whereas CAA is associated with lobar distribution [28]. In this study, when CMBs were categorized according to their locations, the lower serum Cav-1 levels were independently associated with deep or infratentorial CMBs but not with lobar CMBs. These findings might partly indicate that Cav-1 may be involved in the pathogenesis of hypertensive vasculopathy rather than CAA. Recent studies also have revealed that Cav-1 was associated with pulmonary arterial hypertension and was upregulated in high-salt diet-induced endothelial dysfunction and hypertension in type 1 diabetes [29, 30]. However, no evidence demonstrated that there was a relationship between Cav-1 and CAA-related pathology [31], which may partly explain our results.

For ischemic cSVD, there were no remarkable associations between serum Cav-1 levels and SLIs or WMHs in this study. When we repeated the analysis for WMHs with Fazekas 0-1 versus Fazekas 2-3, there was still no significant association (OR = 1.55, 95% CI 0.69–3.46, $P = 0.286$, Model 2). Although CMBs, SLIs, and WMHs are common and representative surrogates of cSVD, there might be differences in risk factors and specific pathophysiology between ischemic and hemorrhagic cSVD [13, 32, 33]. CMBs are more likely to reflect BBB disruption and induced oxidative stress and chronic inflammation, while SILs and WMHs are more likely to be associated with ischemic damage of small perforating arterial or emboli. Some inflammatory biomarker profiles were observed with different levels between hemorrhagic and ischemic MRI surrogates of cSVD [7]. As a plasma membrane cell protein, Cav-1 plays a significant role in regulating endothelial function and BBB permeability. Other experiments revealed that deficiency of Cav-1 appeared atheroprotective and decreased plague area. This phenotype was attributed mainly to defective transendothelial migration of low-density lipoprotein [34, 35]. In the current study, although the levels of serum Cav-1 were higher in SLIs and WMHs groups than those in corresponding negative group of each surrogate, there were no significant differences concerning the levels of serum Cav-1 between patients with SLIs and patients without SLIs or between patients with WMHs and patients without WMHs. This finding deserves confirmation by further studies.

There are limitations which should be emphasized when interpreting the results. First, the sample size is relatively small, which might jeopardize the power of the study, especially when evaluating the effects of CMBs subgroups. Second, blood samples were obtained at one time point within 48 hours of symptom onset. A serial observation of the dynamic changes of Cav-1 levels is lacking. Third, in addition to these typically MRI surrogates, enlarged perivascular space and brain atrophy have been recently suggested as constituents of cSVD, which also deserve further study.

In conclusion, lower serum Cav-1 levels may be associated with CMBs, especially those that are multiple or located in deep brain or infratentorial structures, in patients with acute ischemic stroke. Before being generalized to other populations, these results warrant further studies to establish Cav-1 as a biomarker for predicting CMBs. Observational studies focusing on elderly without stroke and interventional studies in animals (such as gene knockout studies) are needed to determine whether Cav-1 is really a marker of CMBs and a potential target for treating cerebral small vessel disease.

Competing Interests

The authors declare that they have no financial competing interests.

Acknowledgments

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