Melatonin ameliorates cerebral ischemia/reperfusion injury through SIRT3 activation

Lili Liu, Hongping Chen, Jing Jin, Zhanbin Tang, Pengqi Yin, Di Zhong**, Guozhong Li*

Department of Neurology, The First Affiliated Hospital of Harbin Medical University, 23 Youzheng Street, Harbin, 150001, Heilongjiang Province, PR China

ABSTRACT

Aims: Previous literature has shown that melatonin plays a critical role in protecting against cerebral ischemia/reperfusion (I/R) injury. Sirtuin3(SIRT3), as one member of the sirtuin family, protects against oxidative stress-related diseases. However, the association between melatonin and SIRT3 in cerebral I/R injury is not well understood. Our experiment was planned to investigate whether melatonin protects against cerebral I/R injury through SIRT3 activation.

Main methods: We selected transient middle cerebral artery occlusion (tMCAO) mice as the model of cerebral I/R injury. Male C57/BL6 mice were pre-treated with or without a selective SIRT3 inhibitor and then subjected to tMCAO surgery. Melatonin (20 mg/kg) was given to mice by intraperitoneal injection after ischemia and before reperfusion. Then, we observed the changes in the SIRT3 and downstream relative proteins, infarction volume, neurological score, Nissl, H&E and TUNEL staining, and the expression of apoptosis proteins after tMCAO.

Key findings: Melatonin upregulated the expression of SIRT3 after tMCAO, and alleviated the neurological dysfunction and cell apoptosis through SIRT3 activation.

Significance: Our research proved that melatonin promoted SIRT3 expression after tMCAO and alleviated cerebral I/R injury by activating the SIRT3 signaling pathway. This study provides novel therapeutic targets and mechanisms for the treatment of ischemic stroke in the clinic, especially during cerebrovascular reperfusion.

1. Introduction

Stroke is a common disease and the main cause of disability globally [1]. Ischemic stroke, as the most common type of all strokes, occurs when a thrombus blocks the blood vessels that supply blood to the brain [2]. At present, recombinant tissue plasminogen activator (tPA) and rapid vessel recanalization are the preferred therapeutic methods, whenever possible [3–5]. However, because these treatments have the limitation of the narrow therapeutic time window, not all patients can benefit from them. In addition, restoring the blood supply to the brain can paradoxically result in reperfusion injury, which leads to an accumulation of reactive oxygen species (ROS) in brain cells and irreversible brain tissue damage [6]. The most effective therapeutic methods for cerebral I/R injury have not yet been established. Consequently, it is urgent to develop neuroprotective treatments for I/R injury.

Melatonin, a free radical scavenger, is an amine hormone that is generated in most mammals and also in the human pineal gland [7,8].

To date, melatonin has demonstrated diverse pharmacological functions, including anti-oxidative stress properties, circadian rhythm regulation, anti-apoptotic properties, anti-inflammation properties, and others [9,10]. Recent cases have found that melatonin plays an important part in reducing I/R injury, including cerebral I/R injury [11–14]. Accumulating evidence supports the fact that melatonin alleviates I/R injury by reducing cellular oxidative stress, attenuating cellular calcium overload, inhibiting endoplasmic stress, and blocking mitochondrial apoptosis [15,16]. Its functions seem to be widespread and even may have the potential to affect many aspects of human health. However, the underlying intracellular and molecular mechanisms by which melatonin exerts its neuroprotective effects in cerebral I/R injury remain to be further investigated.

Sirtuins are a class of NAD$^+$-dependent histone and non-histone deacetylases, which comprise seven members (SIRT1 to SIRT7) [17]. Sirtuins have been implicated in regulating myriad biological processes, such as cell growth, metabolism, apoptosis, inflammation, and oxidative resistance

Fig. 1. SIRT3 expression is decreased at both the protein and mRNA levels at different time points in tMCAO mice. SIRT3 protein (A) and mRNA (B) levels were detected by Western blot and RT-PCR (n = 4 per group). (C) Immunohistochemistry staining images show SIRT3 expression in the sham and tMCAO groups (n = 4 per group). Data are presented as the mean ± SEM. *P* values were determined by ANOVA followed by the Dunnett test. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; and N.S. not significant, compared to the sham group. Scale bar = 50 μm.

[18]. SIRT3 mainly exists in the mitochondria and has been identified as the most vital deacetylase [19]. Emerging evidence indicates that SIRT3 deacetylates most of the mitochondrial proteins, such as the forkhead box O transcription factor 3 (Foxo3), manganese superoxide dismutase (SOD2), glutamate dehydrogenase (GDH), and cyclophilin D (CypD) [20–23]. Thus, SIRT3 can regulate many cellular biological conditions, including metabolism, mitochondrial dynamics, and cellular oxidative stress by altering the acetylation of the proteins. Recent data have demonstrated that SIRT3 is also associated with ischemic stroke [24,25]. For example, research has revealed that SIRT3 can reduce cerebral tissue injury in a mouse model of experimental stroke by mediating mitochondrial ceramide synthesis [26]. But, whether melatonin alleviates cerebral I/R injury via the mediation of the SIRT3 protein is still unclear.

Thus, our study aimed to investigate the effects of melatonin on SIRT3 after ischemic stroke, and further, to explore its possible neuroprotective mechanism.

2. Materials and methods

2.1. Animal studies

Male C57/BL6 mice weighing 21–24g were purchased from Liaoning Changsheng Biotechnology. Mice were housed in individual coops under a 12h light-darkness cycle and a maintained temperature (24 ± 1 °C). The animals were fed a rodent diet and water and randomly assigned to each experimental group. The experiments were approved by the First Affiliated Hospital of Harbin Medical University in accordance with the guidelines prepared by the National Institutes of Health for the care and use of laboratory animals.

2.2. Construction of the transient middle cerebral artery occlusion model (tMCAO)

Cerebral ischemia was established through the transient middle cerebral artery occlusion (tMCAO) model using a modified intraluminal technique [27,28]. Experimental animals were anesthetized with 10% chloral hydrate (350 mg/kg, intraperitoneally) and their body temperature was maintained at 37°C with an electric blanket during the operation. Then, the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) of the mice were

exposed and isolated under a stereomicroscope. Next, the middle cerebral artery (MCA) was occluded by placing a Doccol suture (diameter = 0.21 mm) into the ECA through the carotid bifurcation to the MCA. Reperfusion was accomplished by removing the suture after 1 h of blockage and then the mice were allowed to recover for 24h, 48h, 3d, 5d, and 7d. The animals in the sham group were subject to the same operation process except that the MCA was occluded. The same person performed all the operations throughout the research.

2.3. Chemical administration

Melatonin (20 mg/kg, M5250, Sigma, USA) and 3-(1H-1,2,3-triazol-4-yl) pyridine (3-TYP, HY-1083, MedChemExpress, USA) [13,29] were solubilized in dimethylsulfoxide (DMSO, less than 2%). Mice in the M + tMCAO and V + tMCAO group were intraperitoneally injected with melatonin or vehicle (DMSO < 2%) after ischemia and before reperfusion. In the 3-TYP + M + tMCAO group and the 3TYP + tMCAO group, mice were intraperitoneally injected with 3-TYP at a dose of 50 mg/kg [29] and every two days mice were given 3-TYP once for a total of three times before the tMCAO surgery. The mice were randomly divided into the following groups: (1) Sham group; (2) tMCAO group; (3) M + tMCAO group; (4) V + tMCAO group; (5) 3-TYP + M + tMCAO group; and (6) 3TYP + tMCAO group.

2.4. Immunohistochemistry, Nissl staining, and H&E staining

After fixing with 4% paraformaldehyde, the brain tissue was embedded with paraffin. The brain tissues were cut into 5-μm-thick sheets for further use. In immunohistochemistry staining, after dewaxing and rehydration, the brain slices were treated with H2O2 (0.3%). Next, after going through antigen retrieval, the brain slices were treated with primary antibody mouse anti-SIRT3 (1:50 dilution, bs-610R, Bioss, USA) at 4 °C overnight, followed by a secondary antibody (PV-6002; Zhongshan Biotechnology, China) for 30 min at room temperature. Finally, the sheets were stained with diaminobenzidine tetrahydrochloride (DAB, ZLI-9018; Zhongshan Biotechnology, China) and observed using a microscope. For the Nissl staining, the sheets were treated with 1% methyl violet and Nissl differentiation liquid. Hematoxylin and eosin were used for HE staining. The tissue sections were examined and photographed under a light microscope.
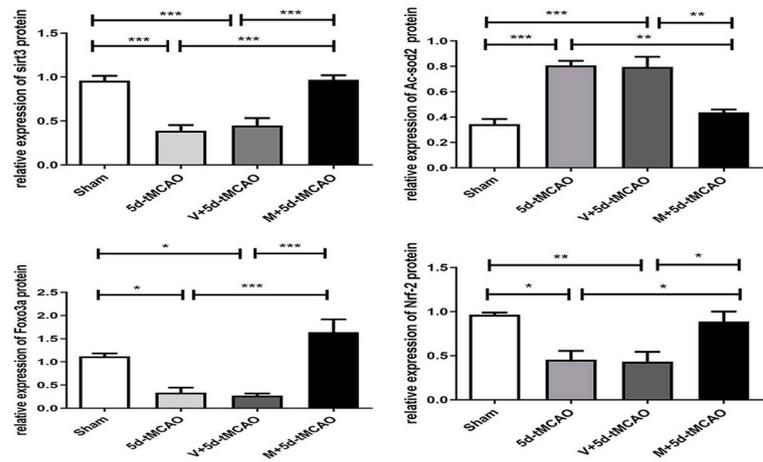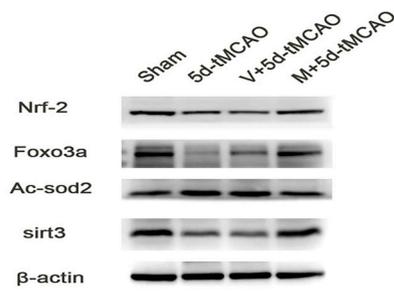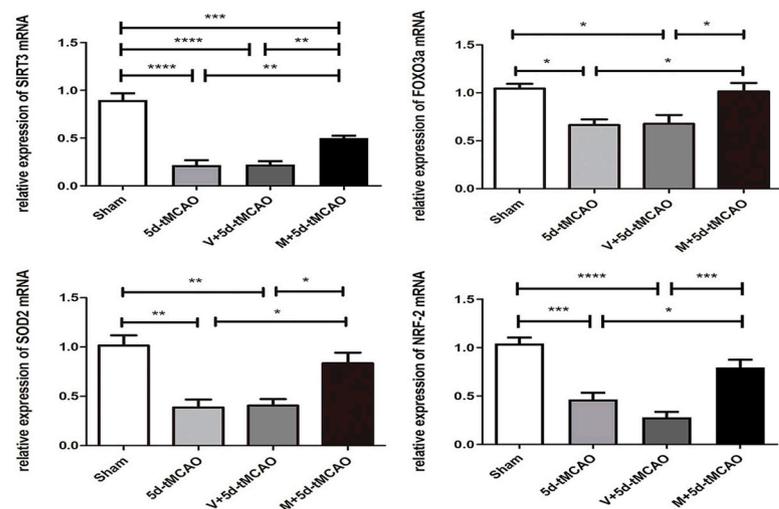
Fig. 2. The SIRT3 and relative protein in the cerebral tissues of mice upon the administration of melatonin after tMCAO. (A) SIRT3, Ac-sod2, Foxo3a, and Nrf-2 protein levels were evaluated by Western blot analysis among groups (n = 4 per group). (B) The mRNA expression of SIRT3, Foxo3a, SOD2, and Nrf-2 were evaluated by RT-PCR among the groups (n = 4 per group). Data are presented as the mean ± SEM. P values were determined by ANOVA followed by the Tukey post hoc test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ and N.S. not significant.

2.5. Apoptosis detection

Cell apoptosis was evaluated in the brain using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. The TUNEL (11684817910; Roche) staining was carried out according to the manufacturer's instructions. In brief, the fixed brain was embedded in paraffin and then cut into 5-μm-thick sections. Then the sections were deparaffinized and rehydrated. After that, the sections were treated with 3% H2O2 and then incubated with sodium citrate (0.1 M) in the microwave for 5 min. After treatment with the TUNEL reaction mixture, the brain sections were stained with DAB. The numbers of TUNEL-positive cells were assessed by counting in five random fields. The apoptotic cell number was considered as the percentage of the positive cells in the total number of cells.

2.6. Measurement of the infarct area

After being sacrificed, the brains were isolated and then were cut into seven sheets beginning from the anterior tip of the frontal lobe (1-mm-thick). The sheets were stained with 2% 2,3,5-triphenyl tetrazolium chloride solution (TTC, Sigma, USA) at 37 °C for 30 min, and fixed in 4% paraformaldehyde. The brain slices were subsequently

photographed with a camera and the infarction volume was evaluated by ImageJ software. The infarction volume(%) = (contralateral hemisphere volume - ipsilateral hemisphere non-infarct volume)/contralateral hemisphere volume × 100%.

2.7. Assessment of neurological deficits

The neurologic deficits tests were performed just before sacrifice and an examiner who was blinded to the treatment conditions evaluated each mouse. The deficits were scored as follows: 0, no deficits; 1, forelimb weakness and torso turning to the ipsilateral side when held by tail; 2, unable to extend the contralateral forelimb; 3, circling to the contralateral side; 4, falling to the contralateral side; and 5, no spontaneous activity or low level of consciousness or death.

2.8. Quantitative real-time PCR

Total RNA was extracted from the right brain by the Trizol reagent (Invitrogen, Thermo Fisher Scientific) and then reverse-transcribed to cDNA via reverse transcriptase (TransGene Biotech) per the manufacturer's instructions. qPCR was carried out with the SuperMix (TransGene Biotech) using the following primers. The primer sequences are as follows:
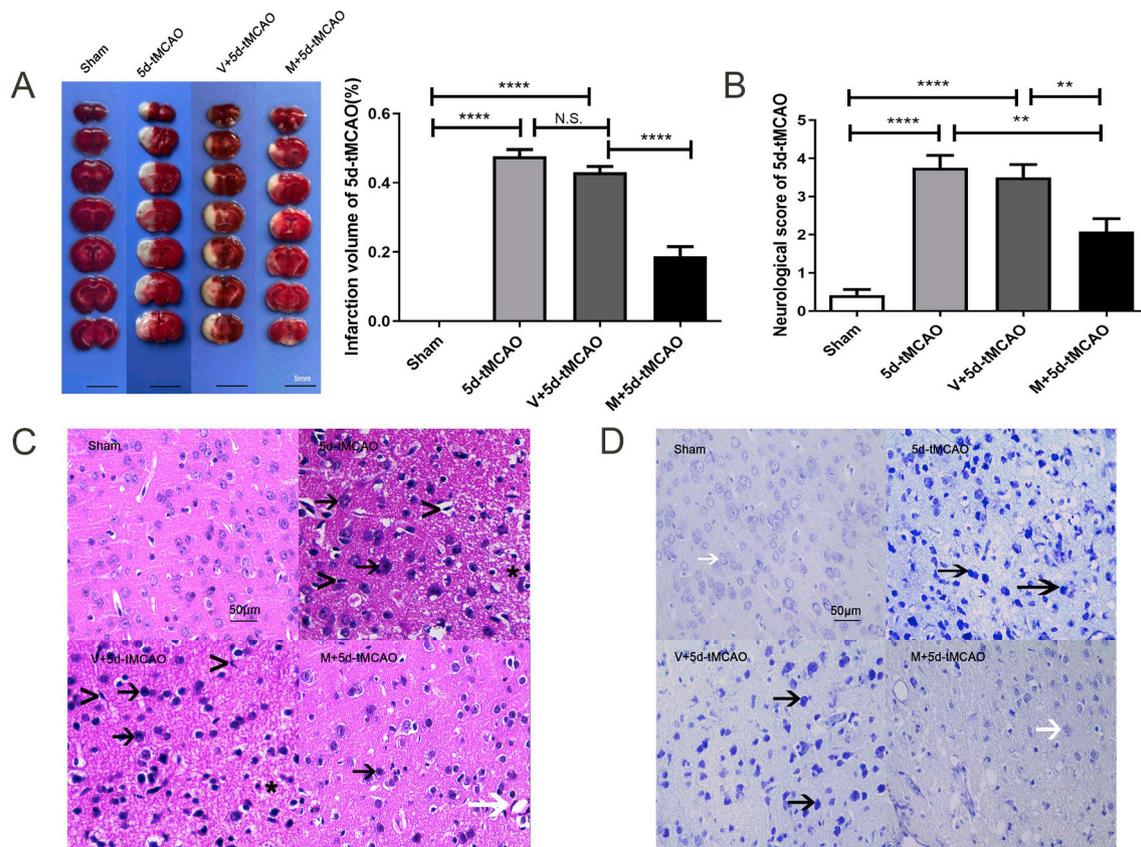
Fig. 3. Melatonin decreased ischemic reperfusion injury after tMCAO. (A) TTC staining of brain tissues five days post-tMCAO (n = 4 per group). Scale bar = 5 mm. (B) Neurological deficit score in mice five days post-tMCAO (n = 12 per group). (C) H&E staining of the mouse brain tissue in the groups (n = 4 per group). In the 5d-tMCAO operation groups, the brain tissue was loose and water had accumulated (*), and microglial cells had increased (>). The black arrow points to the nucleus of neurons that were shrunken and trachychromatic (↑), and in the M + 5d-tMCAO, the white arrow points to vascular hyperplasia. Scale bar = 50 μm. (D) Nissl staining of brain tissues (n = 4 per group). In the sham group, the white arrow points to neurons with Nissl bodies. In the 5d-tMCAO and V + 5d-tMCAO groups, the black arrow points to neurons stained dark with karyopyknosis and Nissl bodies had disappeared. In the M + 5d-tMCAO group, the white arrow points to neurons with Nissl bodies (↑). Scale bar = 50 μm. Data are presented as mean ± SEM. *P* values were determined by ANOVA followed by the Tukey post hoc test, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; and N.S. not significant.

SIRT3, forward 5′-AGTGACATTGGGCCTGTAGTG-3, reverse 5′-TACCCTGAAGCCATCTTTGAA-3′; FOXO3a, forward 5′- CATGGACG ACCTGCTGGATAACATC-3′, reverse 5′-GACGCAAGGAGTTCAGAGACG AAG-3′; SOD2, forward 5′-TGGAGAACCCAAAGGAGAGTT-3′, reverse 5′-GAGCAGGCAGCAATCTGTAA-3′; NRF-2, forward 5′-TCAGTGACTC GGAAATGGAGG-3′, reverse 5′-TATTTTCACATTGGGATTCACGCAT-3′; β-actin, forward 5′-TGGAATCCTGTGGCATCCATGAAAC-3′, reverse 5′-TAAAACGCAGCTCAGTAACAGTCC-3′. Finally, the $2^{-\Delta\Delta Ct}$ method was used to analyze the expression of each gene.

2.9. Western blot analysis

Right-brain tissues were acquired and the protein was extracted on the fifth day of reperfusion. A BCA protein assay kit (Beyotime Biotechnology, China) was used to detect the protein concentrations. In addition, 30 μg of protein from each group was loaded onto a 12% or 10% SDS-PAGE gel. After electrophoresis, brain proteins were transferred onto polyvinylidene difluoride (PVDF) filters. The protein binding was blocked in 5% nonfat milk in TBST for 2 h at room temperature, and then the filters were incubated with primary antibodies at 4 °C overnight. After incubating with goat anti-mouse (1:3000; ZB-2305; Zhongshan Biotechnology, China) or goat anti-rabbit (1:3000; ZB-2301; Zhongshan Biotechnology, China) secondary antibody for 1 h at room temperature, protein bands were washed with TBST and TBS and then visualized by an enhanced chemiluminescence (ECL) reagent (K003, Affinity Biotechnology). The primary antibodies included anti-SIRT3 (1:1000, 5490S, Cell Signaling Technology, Inc., USA), anti-

Foxo3a (1:1000, 10849-1-AP, Proteintech Group, Inc., USA), anti-Nrf-2 (1:1000, 16396-1-AP, Proteintech Group, Inc., USA, anti-Ac-sod2 (1:1000, ab137037, Abcam, USA), anti-Caspase3 (1:1000, 19677-1-AP, Proteintech Group, Inc., USA), anti-Bax (1:1000, 50599-2-Ig, Proteintech Group, Inc., USA), and anti-Bcl-2 (1:1000, 12789-1-AP, Proteintech Group, Inc., USA). β-actin (1:1000, TA-09, Zhongshan Biotechnology, China) was used as an internal contrast. The gray values of all protein bands were quantitatively analyzed by using the Image-Pro Plus 6.0 software.

2.10. Statistical analysis

GraphPad Prism software 8.0 (GraphPad Software, US) was used for the statistical analysis. The data were expressed as mean ± SEM. Differences between groups were performed by one-way ANOVA followed by the Dunnett test or the Tukey post hoc test. A level of $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. SIRT3 expression is downregulated in the mouse brain after tMCAO

To investigate the possible relevance of SIRT3 in cerebral I/R injury, the expression of SIRT3 in the right brain was detected at different time points after tMCAO. The Western blotting (Wb) results revealed a downward in the SIRT3 protein with time; however, we found the downward trend occurred on the third day (*p* = 0.0111, Fig. 1A) and
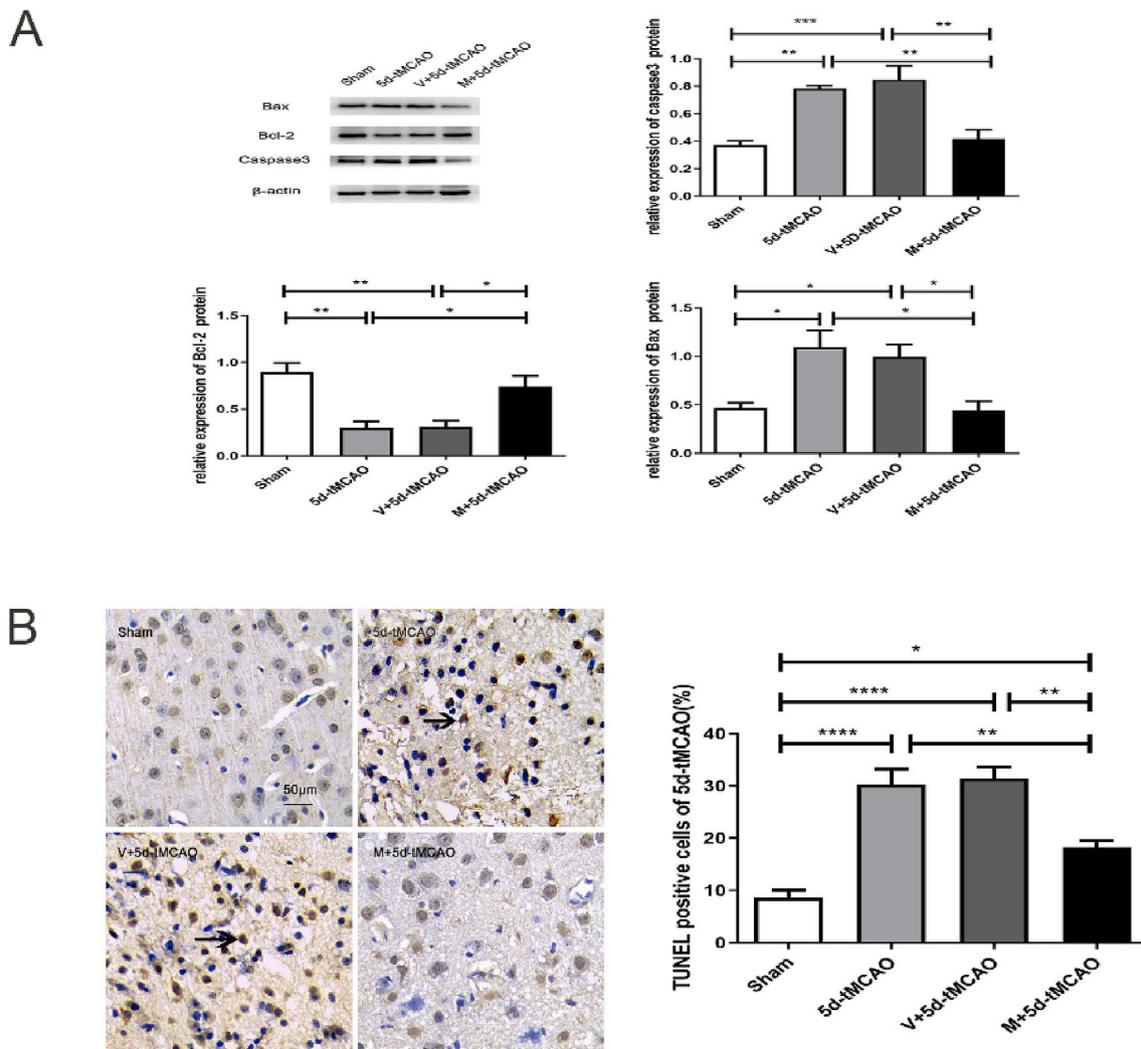
Fig. 4. Melatonin reduced neuronal apoptosis after tMCAO. (A) Relative protein levels of Caspase3 and Bax, Bcl-2 in the sham group, the 5d-tMCAO group, the M + 5d-tMCAO group, and the V + 5d-tMCAO group (n = 4 per group). (B) TUNEL assay of brain tissues (n = 4 per group). The black arrow points to cells with positive staining. Scale bar = 50 μm. Data are presented as the mean ± SEM. *P* values were determined by ANOVA followed by the Tukey post hoc test, *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; and N.S. not significant.

reached its lowest point on the fifth day ($p = 0.0006$, Fig. 1A). In terms of mRNA level, SIRT3 began to decline on the first day ($p = 0.0033$, Fig. 1B) and reached its lowest level on the fifth day ($p < 0.0001$, Fig. 1B) compared to the sham group. Similarly, the same trend in penumbra tissue was observed on immunohistochemistry (Fig. 1C), implying an important role of SIRT3 in cerebral ischemia.

3.2. Melatonin increased SIRT3, Foxo3a, and Nrf-2 expression and decreased Ac-sod2 levels following tMCAO

To investigate the possibility that melatonin provided against cerebral I/R injury via the activation of SIRT3, we examined the expression of SIRT3 and relative proteins after the administration of melatonin. We had previously discovered the changes of SIRT3 in tMCAO and the most obvious change occurred on the fifth day. Thus, to further explore whether melatonin has an effect on the protein expression of SIRT3, different doses of melatonin (10 mg/kg, 20 mg/kg, 30 mg/kg, 50 mg/kg) were given after ischemia and before reperfusion, and the relative expression of SIRT3 in the cerebral tissue was evaluated. Our Wb results revealed that the SIRT3 protein expression was increased after tMCAO when the mice were given melatonin 20 mg/kg ($p = 0.0190$ vs 5d-tMCAO, Fig. S1). Therefore, this dose was chosen for subsequent experiments.

Based on the results in Fig. 2A, compared with the sham group, 5d-tMCAO decreased the expression of the SIRT3 protein ($p = 0.0003$) and promoted the acetylation of SOD2 (Ac-sod2) ($p = 0.0001$). Moreover, 5d-tMCAO decreased the protein expression of Foxo3a ($p = 0.0171$) and Nrf-2 ($p = 0.0119$) in the mouse brain. Compared to the V + 5d-tMCAO group, Western blotting demonstrated that melatonin increased the protein expression of SIRT3 ($p = 0.0006$), Foxo3a ($p = 0.0002$), and Nrf-2 ($p = 0.0238$), and decreased Ac-sod2 ($P = 0.0013$) expression. Furthermore, we explored the mRNA level of SIRT3, Foxo3a, Nrf-2, and SOD2, and found that, compared to the sham group, 5d-tMCAO decreased the mRNA expression of SIRT3, Foxo3a, Nrf-2, and SOD2 (Fig. 2B, $p < 0.0001$, $p = 0.0150$, $p = 0.0003$, $P = 0.0017$). At the same time, we found melatonin administration upregulated SIRT3, Foxo3a, Nrf-2, and SOD2 expression at the transcription level (Fig. 2B, $p = 0.0091$, $p = 0.0311$, $p = 0.0007$, $p = 0.0246$ vs. V + 5d-tMCAO group, respectively). This findings clearly demonstrated that melatonin attenuated the cerebral ischemia/reperfusion injury by regulating SIRT3 and its relative molecules.

3.3. Melatonin decreased cerebral ischemia/reperfusion injury after tMCAO

To illustrate the possible neuroprotective effect of melatonin, we measured the neurological deficit score, infarct volume, and
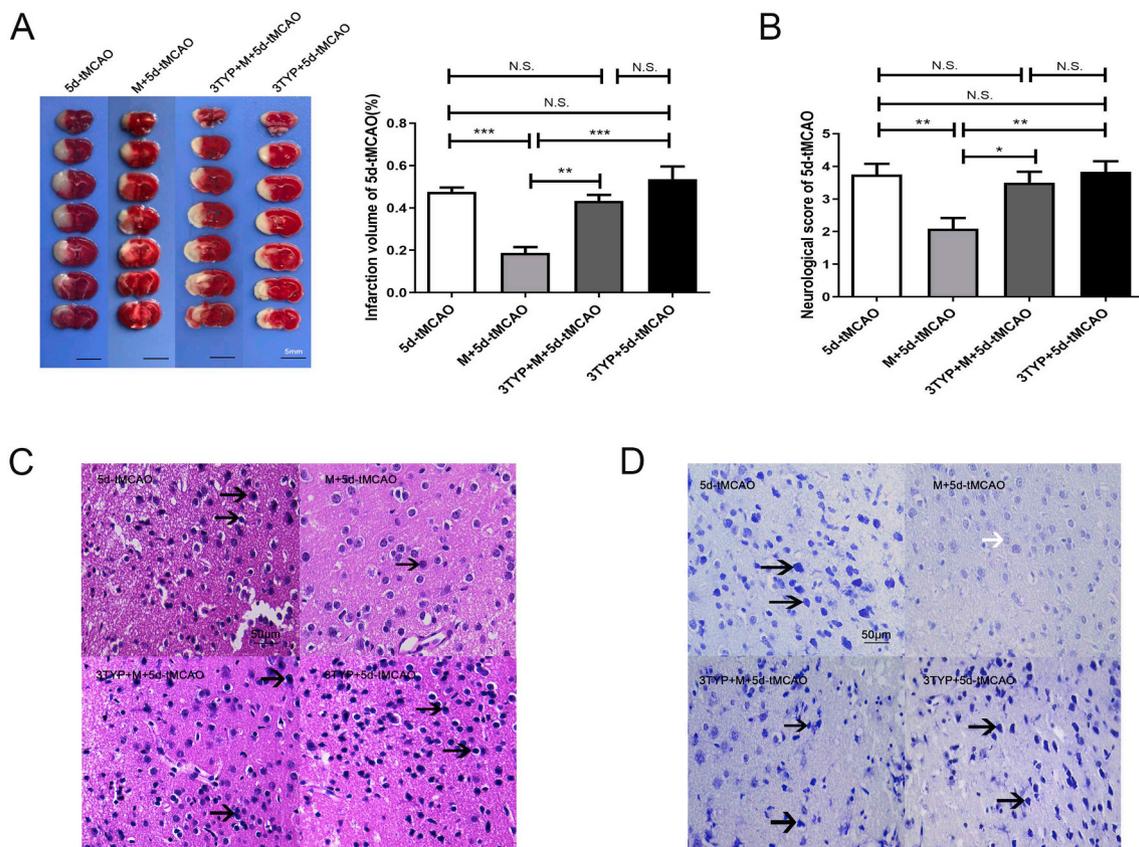
Fig. 5. Pretreatment with 3-TYP abolished the cerebral-protective effects of melatonin by increasing the infarct volume, and neurological deficit scores *in vivo*. 3-TYP (50 mg/kg, i.p.) was given every two days for a total of three times before tMCAO surgery. (A) TTC staining of brain tissues five days post-tMCAO (n = 4 per group). Scale bar = 5 mm. (B) Neurological deficit score in mice five days post-tMCAO (n = 12 per group). (C) H&E staining of the mouse brain tissue in the groups (n = 4 per group). The black arrow points to the nucleus of neurons that were shrunken and trachychromatic (↑). Scale bar = 50 μm. (D) Nissl staining of brain tissues (n = 4 per group). In the 5d-tMCAO, 3TYP + M + 5d-tMCAO and 3TYP + 5d-tMCAO groups, the black arrow points to neurons stained dark with karyopyknosis and Nissl bodies had disappeared. In the M + 5d-tMCAO group, the white arrow points to neurons with Nissl bodies (↑). Scale bar = 50 μm. Data are presented as mean ± SEM. *P* values were determined by ANOVA followed by the Tukey post hoc test, *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; and N.S. not

morphological changes in brain tissue after five days of reperfusion. As we can see in Fig. 3A, melatonin apparently decreased the infarction volume in the mouse brain compared to the V + 5d-tMCAO group (p < 0.0001). Meanwhile, melatonin treatment also reduced the neurological score in mice (Fig. 3B p = 0.0086). Brain morphological examination was performed after tMCAO. H&E staining showed that the tissues of the infarct zone were porous, and that the nucleus of the neurons around the infarction were pyknotic and dyed more deeply, the penumbra tissue was swollen, and more microglial cells and vascular hyperplasia appeared after tMCAO (Fig. 3C). However, H&E staining demonstrated that less degenerated neurons with deeply stained nuclei were in the penumbra of the M + 5d-tMCAO mice. Meanwhile, the Nissl bodies disappeared more in the neurons of the V + 5d-tMCAO compared to the M + 5d-tMCAO group (Fig. 3D). The results enabled us to believe that melatonin had neuroprotective effects in the brain against ischemia/reperfusion injury.

3.4. Melatonin reduced neuronal apoptosis after tMCAO

As is known, apoptosis is one of the vital pathophysiological processes in I/R injury, thus, we next explored the potential influence of melatonin on apoptosis. Our Western blotting data demonstrated that the Caspase 3 protein (p = 0.0028) and Bax protein (p = 0.0133) were significantly increased and Bcl-2 protein (p = 0.0031) was reduced in the 5d-tMCAO mice compared to the sham group (Fig. 4A). However, the melatonin treatment down-regulated the Caspase 3 protein (p = 0.0018) and Bax protein expression (p = 0.0287), and, at the same

time, sharply improved the Bcl-2 protein expression (p = 0.0272) after tMCAO (Fig. 4A). In addition, more apoptosis cells appeared in the V + 5d-tMCAO group than in the M + 5d-tMCAO, as evidenced by the TUNEL assay (Fig. 4B, p = 0.0024). Collectively, our data revealed that melatonin exerted its protective effect through anti-apoptosis in cerebral I/R injury.

3.5. Melatonin mitigates cerebral I/R injury via the SIRT3 signaling pathway

We used the 3-TYP, a SIRT3 inhibitor, to prove that melatonin reduced cerebral I/R injury via the activation of SIRT3. Previous research has found that 3-TYP has little influence on the mouse brain [29]. Based on the data in Fig. 5, we found that 3-TYP did not influence the brain infarct volume (Fig. 5A, p = 0.6665) and the neurological deficit scores, compared to the 5d-tMCAO group (Fig. 5B, p = 0.9980). But, we found that 3-TYP administration attenuated the protective effect of melatonin by increasing infarct volume (Fig. 5A, p = 0.0025) and increasing the neurological deficit scores compared to the M + 5d-tMCAO group (Fig. 5B, p = 0.0207). At the same, there was little difference in the morphological changes between the 3TYP + 5d-tMCAO group and the 5d-tMCAO group (Fig. 5C, D). However, H&E staining demonstrated that more degenerated neurons with deeply stained nuclei were displayed in the penumbra of the 3TYP + M + 5d-tMCAO group (Fig. 5C). In addition, the Nissl bodies disappeared more often in the neurons of the 3TYP + M + 5d-tMCAO group compared to the M + 5d-tMCAO group (Fig. 5D). These results demonstrated that melatonin mitigated
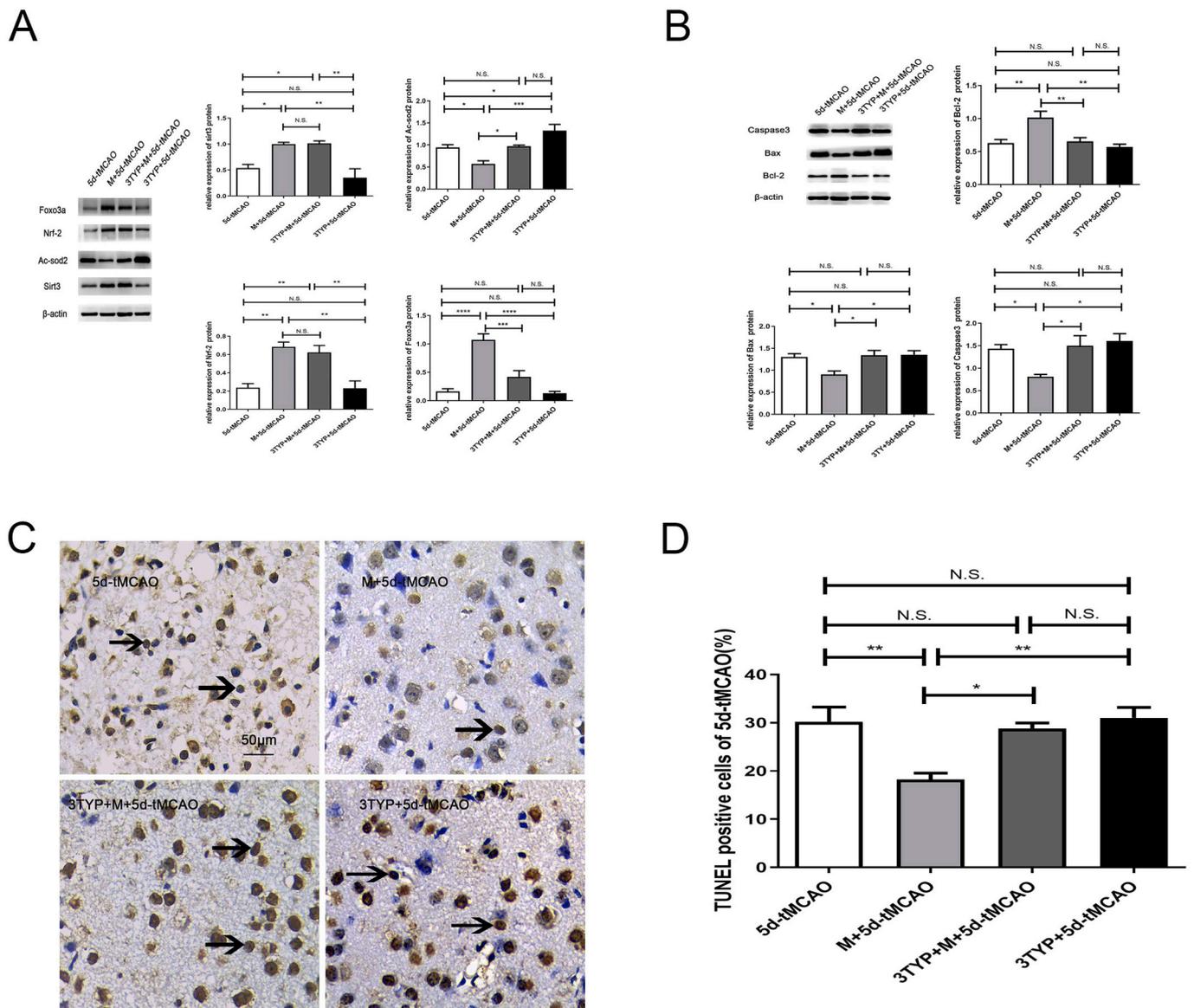
Fig. 6. Pretreatment with 3-TYP abolished the anti-apoptotic effects of melatonin following tMCAO. (A) The relative protein levels of SIRT3, AC-SOD2, Foxo3a, and Nrf-2 in the 5d-tMCAO group, the M + 5d-tMCAO group, the 3TYP + M + 5d-tMCAO group, and the 3-TYP + 5d-tMCAO group (n = 4 per group). (B) Relative protein levels of Caspase3, Bax, and Bcl-2 in the 5d-tMCAO group, the M + 5d-tMCAO group, the 3-TYP + M + 5d-tMCAO group, and the 3TYP + 5d-tMCAO group (n = 4 per group). (C, D) TUNEL assay of brain tissues (n = 4 per group). The black arrow points to cells with positive staining. Scale bar = 50 μm. Data are presented as mean ± SEM. *P* values were determined by ANOVA followed by the Tukey post hoc test, *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; and N.S. not

tMCAO-induced brain damage via regulating the SIRT3 signaling pathway.

3.6. Melatonin mitigates apoptosis in cerebral I/R injury through the SIRT3 signaling pathway

Based on the results in Fig. 6, we found that there was little difference in the expression of the SIRT3 protein between the 5d-tMCAO group and the 3-TYP + 5d-tMCAO group, but the acetylation of SOD2 level was upregulated in the 3TYP + 5d-tMCAO group compared to the 5d-tMCAO group (Fig. 6A, p = 0.5549, p = 0.0353). This indicated that 3-TYP did not affect the SIRT3 protein expression, but could restrain the deacetylase activity of SIRT3. This result was consistent with previous research [13]. Meanwhile, we found 3-TYP had little effect on the Foxo3a, Nrf-2, Bcl-2, Caspase3 and Bax protein expression compared to the 5d-tMCAO group (Fig. 6A, p = 0.9888, p = 0.9999, Fig. 6B, p = 0.9098, p = 0.8310, p = 0.9761). In Fig. 6, we found that the administration of 3-TYP did not change the up-regulation of the SIRT3

protein caused by melatonin, but the level of acetylation of SOD2 was upregulated compared to the M + 5d-tMCAO group (Fig. 6A, p = 0.9997, p = 0.0269). The Foxo3a proteins were decreased in the 3-TYP + M + 5d-tMCAO group compared to the M + 5d-tMCAO group (Fig. 6A, p = 0.0003). Interestingly, the expression of Nrf-2 showed no significant difference between the 3-TYP + M + 5d-tMCAO group and the M + 5d-tMCAO group (Fig. 6A, p = 0.9228). In addition, the administration of 3-TYP abolished the anti-apoptotic function of melatonin. This was because that the Wb results showed that Bcl-2 protein expression was decreased and Bax and Caspase-3 protein expression were increased in the 3TYP + M + 5d-tMCAO group compared to the M + 5d-tMCAO group (Fig. 6B, p = 0.0092, p = 0.0217, p = 0.0265). In addition, the intraperitoneal injection of 3-TYP increased the number of apoptosis cells, as shown by the TUNEL assay, compared to the M + 5d-tMCAO group (Fig. 6C, D p = 0.0113). Taken together, all these data ensured that melatonin reduced apoptosis through the SIRT3 signaling pathway in cerebral I/R injury.

4. Discussion

In our present research, melatonin administration during tMCAO conferred a protective effect in the mouse brain. Based on our results, we can see that melatonin improved post-ischemic neurological functional recovery, reduced brain infarct volume, and reduced the apoptotic rate of brain cells. These protective functions of melatonin might be partially attributable to the activation of SIRT3 because selective SIRT3 inhibitor administration remarkably eliminated these protective functions of melatonin in cerebral ischemia/reperfusion injury. Our study found that melatonin promoted SIRT3 expression after tMCAO and alleviated cerebral I/R injury by activating the SIRT3 signaling pathway.

For the last decade, vascular recanalization therapy has been widely incorporated into clinical practice, and early reperfusion treatment has been confirmed to be the most efficient way to improve long-term outcomes for ischemic stroke patients [5]. However, the simultaneous production of additional ROS during reperfusion can further aggravate brain damage, resulting in enlarged infarct size and neurological deterioration [6]. Some researchers use melatonin to ameliorate ischemic reperfusion injury because it has free radical scavenging and antioxidant functions [11,13,30–33]. In addition to being a highly effective, direct free-radical scavenger and an indirect antioxidant, melatonin has other characteristics that warrant further research. For example, melatonin is easily absorbed through any means of administration, it can easily pass through all morphological and physiological obstacles, such as the placenta and the blood-brain barrier, it seems to enter all parts of each cell where it can thwart oxidative damage, and it has low toxicity [34]. Experimental findings discovered that melatonin confers important protective functions in many conditions, including aging, cardiovascular diseases, neurodegenerative diseases, diabetes, and metabolic syndrome [35,36]. In addition, researchers have also reported that sirtuins are related to brain aging and neurodegenerative disorders [37], including ischemic brain injury. For example, research has indicated that SIRT1 had a neuroprotective role in cerebral ischemia through the deacetylation of p53 and NF-κB [38]. SIRT3 can protect neurons from N-methyl-D-aspartate (NMDA)-induced excitotoxicity during ischemic brain damage [39]. Hence, the connection between melatonin and sirtuins in cerebral I/R injury inspired our interest.

Of note, Venegas and coworkers found that, at least for brain cells, melatonin was unequally distributed within the subcellular structure, with brain mitochondria having a much higher melatonin concentration than other cell sites [8]. SIRT3 is a significant deacetylase, which influences many proteins to regulate mitochondrial action and ROS generation [20]. Dai SH and colleagues demonstrated that SIRT3 protected neurons from oxygen and glucose deprivation (OGD) damage by modulating the AMPK-mTOR pathway to induce autophagy [40]. We were more concerned with the connection between melatonin and SIRT3 in cerebral I/R injury. In our study, we detected the expression of SIRT3 in the ipsilateral brain tissue at different time points after tMCAO (Fig. 1). Our results revealed that SIRT3 was significantly decreased with time after tMCAO. Klishadi MS also found that myocardial I/R injury led to a marked fall in the expression of SIRT3 [41]. In addition, SIRT3 downregulation might enhance the susceptibility of cardiomyocytes and the heart to I/R damage [42]. Previous research has indicated that overexpression of SIRT3 reduced cerebral I/R damage through the regulation of the Wnt/β-catenin pathway and by blocking mitochondrial division [43]. Moreover, restoring SIRT3 expression and activity through administration of the AMPK agonist or an antioxidant agent improved cisplatin-induced acute kidney injury [44]. Similarly, our data showed an apparent decline in SIRT3 expression, which might enhance the oxidative stress damage in the brain.

To investigate the connection between melatonin and SIRT3 in cerebral I/R injury, we gave mice melatonin after ischemia and reperfusion during tMCAO surgery. We found that the expression of SIRT3 was increased (Fig. 2) compared to that in the tMCAO group, whether at the protein level or at the transcription level. Furthermore, we found the expression of Foxo3a and Nrf-2 were also increased at the protein and transcription levels, and that the Ac-SOD2 protein was decreased and the mRNA expression of SOD2 was increased (Fig. 2) at the same time. Most of the proteins in the mitochondria are deacetylated to exert their biological functions. SIRT3 is one of the several very important deacetylases in the mitochondria [18,19]. The SOD2 localized in the mitochondria is one main target of SIRT3, which has been widely detected in oxidative stress damage [45]. SOD2 plays an important role in maintaining the redox balance of the cell, and the abnormal regulation of SOD2 has been closely related to various diseases [46,47]. The elevation of lysine acetylation of SOD2 decreases SOD2 enzymatic activity, and recent studies have shown that SIRT3 specifically regulated SOD2 activity through deacetylation at lysine68 [48,49]. Chen Y and co-workers found that SIRT3 regulated ROS clearance by decreasing the acetylation of SOD2 in tumors [50]. As shown in a study by Jiang He et al., the upregulation of SIRT3 expression improved the re-endothelialization capacity of endothelial progenitor cells via increasing SOD2 deacetylation and subsequently reducing mitochondrial oxidative stress in hypertension [51]. Foxo3a is a forkhead transcription factor and one important member of the FOXO family. Foxo3a is another target of SIRT3, which can regulate the expression of genes involved in stress resistance, longevity, apoptosis, and autophagy [21,52]. Previous research has confirmed that Nrf2 could upregulate SIRT3 expression after melatonin administration [53]. In this study, we observed that the protective effect of melatonin might be mediated by SIRT3, Foxo3a, Nrf-2, SOD2, and Ac-SOD2. Taken together, the relationship between SIRT3, Nrf-2, Foxo3a, SOD2, and Ac-SOD2 needs to be investigated in further research. In our research, we found that melatonin decreased ischemia-reperfusion injury, as confirmed by the decrease in the infarction volume, neurological deficit scores, and abnormally stained nerve cells by H&E and Nissl staining (Fig. 3). Furthermore, the administration of melatonin decreased the pro-apoptotic factors Caspase-3 and Bax expression, and increased anti-apoptotic factor Bcl-2 expression compared to the tMCAO group (Fig. 4). These data indicated that melatonin played neuroprotective effect role through its anti-apoptotic properties. This result is consistent with previous research. For example, Rancan L found that melatonin treatment reduced the apoptotic response in aged rats after brain ischemia [54]. Melatonin treatment up-regulated the Bcl-2 protein level during transient focal ischemia [55] and down-regulated the generation of apoptosome. As shown in the study by KilicE, melatonin treatment improved neuronal survival and reduced striatum cell damage related to Caspase-3 suppression in MCAO mice [56]. Melatonin could block the activation of the apoptotic caspase cascade and then could decrease the pBad levels when mice were pretreated with melatonin (5 mg/kg) [57]. In a study by Bochaton Tiong YLand Ng KY, their results suggested that melatonin could alleviate high glucose-induced apoptosis in Schwann cells [54]. Thus, it seems that these results confirm the predominant anti-apoptotic effect of melatonin through various mechanisms.

Finally, to further illustrate whether melatonin exerts its anti-apoptotic and neuroprotective functions through SIRT3 activation, we used the selective SIRT3 inhibitor, 3-TYP, to test this hypothesis. 3-TYP diminished the neuroprotective function of melatonin (Fig. 5/6). These results indicated that SIRT3 participated in the protective action of melatonin against cerebral I/R injury. Previous studies have verified that melatonin displayed a protective role in ameliorating the age-induced fertility decline [58], TNF-α-mediated hepatocyte damage [59], cadmium-induced hepatotoxicity [23], and diabetic cardiomyopathy [60], all of which are regulated by SIRT3. Melatonin provided protection from the effects of early brain damage following subarachnoid hemorrhage in mice by increasing the expression of murine SIRT3 [61]. SIRT3 overexpression could activate the NF-κB pathway and then could increase the ratio of Bcl-2 to Bax, possibly explaining why SIRT3 could protect H9c2 cells from apoptosis [62]. In normal conditions,

deacetylated Ku70 suppresses the translocation of Bax to the mitochondria, and then blocks apoptosis [63]. SIRT3 was recently found to deacetylate Ku70, and this enhanced the interaction of Ku70 with Bax, and thus, exerted an anti-apoptotic function [64]. In our study, we also found that when the activity of SIRT3 was inhibited, the apoptosis in the brain was increased, and, notably, the expression of the Foxo3a protein was decreased. However, under the same conditions, Nrf-2 was not affected. Therefore, we speculate that melatonin protects against cerebral I/R injury by regulating the SIRT3-Foxo3a pathway through the mediation of Nrf-2. Previous research has shown that SIRT3 inhibited prostate cancer metastasis via the regulation of Foxo3a by inhibiting the Wnt/β-catenin pathway [65]. Ruankham W observed that sesamin and sesamol activated SIRT1-SIRT3-Foxo3a expression, and then inhibited Bax expression, upregulated the Bcl-2 protein expression, and ultimately protected human neuronal cells from H_2O_2 -induced oxidative stress [66]. Previous studies have shown that Nrf2 could directly upregulate SIRT3 expression [53,67]. In the present study, we explored only the effect of SIRT3 in anti-cerebral I/R injury after the administration of melatonin *in vivo*. However, the mechanisms by which melatonin affects SIRT3 and by which SIRT3 regulates Foxo3a following cerebral I/R are far from clear and additional studies will need to elucidate this.

In general, this study provides new therapeutic pathways and research perspectives for the treatment of cerebral I/R injury. Our data revealed that melatonin ameliorated cerebral I/R injury, possibly through SIRT3 signaling pathway activation. We found that melatonin could elevate the protein expression of both Foxo3a and Nrf-2 in tMCAO mice. However, the specific mechanisms of molecules upstream and downstream of SIRT3 require further investigation.

5. Conclusion

In conclusion, our study revealed that melatonin treatment protects against neurological dysfunction and apoptosis through the activation of the SIRT3 signaling pathway. Our data provide new potential therapeutic targets and mechanisms for the therapy of cerebral I/R damage. Furthermore, our study provides powerful supporting evidence for clinical trials on melatonin supplementation as an early intervention to ameliorate I/R injury in clinical ischemic stroke patients.

Authors' contributions

Lili Liu designed the study and wrote the paper. Guozhong Li and Di Zhong designed the research and critically reviewed the manuscript. Lili Liu, Hongping Chen, Jing Jin, Zhanbin Tang, and Pengqi Yin performed the research. All authors have read and approved the article.

Declaration of competing interest

No conflict or financial interests.

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Abbreviations

I/R	ischemia/reperfusion
SIRT3	Sirtuin3
ROS	reactive oxygen species
tMCAO	transient middle cerebral artery occlusion
CCA	common carotid artery
ECA	external carotid artery
ICA	internal carotid artery
MCA	middle cerebral artery
Foxo3a	forkhead box O transcription factor 3a
SOD2	manganese superoxide dismutase
Nrf2	nuclear factor erythroid 2-related factor 2
Bcl-2	B-cell lymphoma-2
3-TYP	3-(1H-1,2,3-triazol-4-yl) pyridine
H&E	hematoxylin and eosin
DAB	diaminobenzidine tetrahydrochloride
TTC	2, 3, 5-triphenyl tetrazolium chloride.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.117036.

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