Experimental Animals
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Original

# Transmembrane p24 trafficking protein 10 (TMED10) inhibits mitochondrial damage and protects neurons in ischemic stroke via the c-Jun N-terminal kinase (JNK) signaling pathway

Qiushi LI[1, 2], Xuying LIU[3], Ruixian XING[2] and Rubo SUI[1,2]

[1]*Medical College of Soochow University, No. 1, Shizi Street, Suzhou, Jiangsu Province, 215021, P.R. China*
[2]*Department of Neurology, The First Affiliated Hospital of Jinzhou Medical University, No. 2, Section 5, Renmin Street, Jinzhou, Liaoning Province, 121000, P.R. China*
[3]*Department of Neurology, The Third Affiliated Hospital of Jinzhou Medical University, No. 2, Section 5, Heping Road, Linghe District, Jinzhou, Liaoning Province, 121000, P.R. China*

**Abstract:** Stroke, a type of acute cerebrovascular disease, is a global disease with high mortality. Neuronal ischemia and hypoxia are closely related to occurrence and development of cognitive impairment. Transmembrane p24 trafficking protein 10 (TMED10) as a transmembrane protein involves in vesicle protein transport in the secretory pathways. However, the function and mechanism of TMED10 on ischemic stroke and cognitive impairments remain unclear. In current study, TMED10 was highly expressed in cerebral ischemic penumbra of middle cerebral artery occlusion (MCAO) mouse model. Downregulation of TMED10 suppressed cell survival and facilitated apoptosis in primary cortical neurons, which were grown under oxygen glucose deprivation/reoxygenation (OGD/R) condition. Upregulation of TMED10 protected neurons form apoptosis induced by OGD/R. Further research indicated that the decrease of TMED10 resulted in neuronal mitochondrial injury through increasing reactive oxygen species (ROS) production. Meanwhile, TMED10 reduction induced neuronal apoptosis and mitochondrial damage through activating the c-Jun N-terminal kinase (JNK) pathway. Moreover, the knockdown of TMED10 increased cerebral infarction area, aggravated neuronal injury and promoted neuronal apoptosis through activating the JNK pathway in the cerebral ischemic penumbra of MCAO mouse model. Additionally, Morris water maze test verified that the severity of cognitive impairment increased with the decline of TMED10. Collectively, this study reveals that TMED10 inhibits mitochondrial damage, and protects neurons from apoptosis in MCAO-induced ischemic stroke and cognitive impairment via blocking the JNK pathway.
**Key words:** apoptosis, cerebral ischemic stroke, cognitive impairment, c-Jun N-terminal kinase (JNK) pathway, transmembrane p24 trafficking protein 10 (TMED10)

## Introduction

Stroke is a cerebrovascular disease caused by a defect in cerebral artery. It is the most common life-threatening neurological disorder, including ischemic stroke and hemorrhagic stroke. Ischemic stroke is a high incidence of cerebral disease caused by cerebral thrombosis blocking brain arteries [1]. In both developed and developing countries, it is one of the leading causes of neurological morbidity and mortality [2]. Cerebral ischemia causes nerve cells to lack oxygen and certain essential nutrients, which can lead to brain damage and even neuronal apoptosis [3, 4]. In addition, some studies and patient data have shown a strong association between stroke and cognitive impairment [5]. More than one-third of stroke patients may develop cognitive impairment, and cannot

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*Corresponding author: R. Sui.email: sui_jzykdx2022@yeah.net*
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be diagnosed until at least 6 months later. Thus, post-stroke cognitive impairment is considered to be one of the major complications of ischemic stroke [6, 7]. Therefore, developing potential therapeutic targets and exploring the pathogenesis of cerebral ischemia is especially important in attenuating of brain stroke and cognitive impairment.

Oxidative stress refers to the imbalanced state of oxidation and antioxidant. Several studies have indicated that oxidative stress is the main factor of cerebral ischemia stroke [8]. In normal physiological environments, reactive oxygen species (ROS) are produced during aerobic metabolism [9]. When the environment become hypoxia and ischemia, excessive ROS production leads to cell necrosis and apoptosis through affecting the DNA and protein synthesis or mitochondrial dysfunction [3]. ROS overproduction increases the opening of mitochondrial permeability transition pores and reduces mitochondrial membrane potential, which results in neuronal apoptosis [10]. Some studies have demonstrated that ROS can activate the c-Jun N-terminal kinase (JNK) pathway, which participates in promoting mitochondrial damage and apoptosis in cerebral ischemia models [11, 12]. In addition, mitochondria mediated pathways are important participants in inducing apoptosis, including the activation of the JNK pathway, and the massive escape of cytochrome c from mitochondria leads to the mitochondrial dysfunction [13]. Based on the above, it is an effective treatment for ischemic stroke to explore molecular markers that improve oxidative stress and mitochondrial damage.

Transmembrane p24 trafficking protein 10 (TMED10), also known as transmembrane protein with type I topology 21 (TMP21), is a member of the p24 transporter receptor family [14]. As a transmembrane protein [15], it involves in the transport of vesicle proteins in the secretory pathways [16, 17] and has been verified to be expressed in the tissues such as heart, lung, liver, adrenal gland and brain [18]. TMED10, a member of the γ-secretase complex, is down-regulated in brain tissues of patients with Alzheimer disease (AD) [19, 20]. Shin *et al.* have demonstrated that TMED10 as an activator of the mTOR pathway inhibits ROS production and the autophagy of SH-SY5Y cells [21]. It has been reported that TMED10 attenuated apoptosis in prostate cancer cell lines by blocking the activation of the JNK pathway [22, 23]. Moreover, TMED10 expression was increased in the penumbra area of cerebral cortex after 4h or 24h of infarction in the cerebral ischemia model induced by local photochemistry [24]. However, the function and regulation mechanism of TMED10 in ischemic stroke and cognitive impairment are not fully understood.

The present study aims to reveal the character of TMED10 in ischemic stroke and cognitive impairment. We found that the downregulation of TMED10 suppressed the viability of primary cortical neurons and enhanced oxidative stress, mitochondrial injury and cell apoptosis *in vitro*. Besides, the middle cerebral artery occlusion (MCAO)-induced neuronal injury and cerebral ischemia stroke was aggravated by the TMED10 reduction via the JNK signaling pathway *in vitro* and *in vivo*. Furthermore, we verified that the knockdown of TMED10 would exacerbate the cognitive impairment. This study provides potential options for the remission of ischemic stroke and cognitive impairment.

## Materials and Methods

### Cell culture

The isolation and culture of primary cortical neurons refer to the research of Yuan *et al.* [25] The cerebral cortex tissues were collected from the newborn C57BL/6J mice. They were sliced into small pieces and digested with 5 ml 0.25% trypsin (Sigma, St. Louis, MO, USA) at 37°C for 15 min. The mixture was centrifuged and cleaned with Neurobasal culture medium (Gibco, Grand Island, NY, USA), then seeded in culture plates covered by 0.05% polylysine and cultured in an incubator with 5% $CO_2$ at 37°C for 4–6 h. The medium was replaced every 2–3 days, and the cells were photographed and morphologically identified after 7 days of culture.

TMED10 deficiency in primary cortical neurons was established with lentiviral vectors carrying sh-TMED10-1 (sh-1) and sh-TMED10-2 (sh-2) respectively. The short hairpin RNA (shRNA) targeting TMED10 and negative scrambled shRNA (sh-NC) were synthesized from General Biol. (Anhui, China). The sequences of shRNAs are as follows. sh-NC: 5′-TTCTCCGAACGTGTCACGT-3′, sh-1: 5′-GGAAGACTATGACATGTTTGA-3′, sh-2: 5′-CCTACGAGATCACCGACCAGT-3′. To verify the effect of TMED10 upregulation on neurons, coding sequence (CDS) of TMED10 was cloned into the plasmid PLJM1-EGFP-puro (Fenghui Biotechnology Co., Ltd., Hunan, China). After lentiviral packaging, concentrated lentivirus carried sh-1, sh-2, sh-NC, OE-vector or OE-TMED10 was stored at −80°C.

The brief of lentivirus infection is as follows. Primary cortical neurons were plated at $1.0 \times 10^5$ cells/well in 6-well plates. When neurons were grown to 70% confluency, the concentrated lentivirus was added to the culture media (MOI=10). They were then cultured in an incubator with 5% $CO_2$ at 37°C for 72 h. To established the oxygen glucose deprivation/reoxygenation (OGD/R) model, the neurons were transferred to an incubator that

maintained 94% N2, 5% $CO_2$ and 1% $O_2$. After 1 h of culture in the anoxic environment, the cells were cultured in the normal environment for 24 h. In addition, to verify whether the JNK pathway involves in the neuronal mitochondrial damage mediated by TMED10, cells were treated with 5 $\mu$M SP600125 (Shyuanye, Shanghai, China) for 30 min. Then, the cells were grown under OGD/R condition.

## Animals

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals. All protocols were approved by the Ethics Committee of The Third Affiliated Hospital of Jinzhou Medical University. Male C57BL/6J mice aged 8 weeks were exposed to an environment whose temperature was 22 ± 1°C, humidity was 45–55% and 12 h light/dark cycle every day. They have free access to food and water. The MCAO model was established after one week of adaptive feeding.

Experiment 1: The mice were subjected to sham or 60-min MCAO surgery. The protocol of model establishment was referred to the previous studies [26, 27]. Briefly, after the mice were anesthetized, the middle cerebral artery (MCA) blood flow was blocked by the suture, which was withdrawn after 60 min to allow reperfusion. Then the MCAO model was confirmed by the laser Doppler. The sham group performed the same procedure except for blocking the MCA. After 24 h, the brain tissues were collected and partially fixed by 4% paraformaldehyde. Part of them were frozen in liquid nitrogen and stored in −70°C ultra-low temperature refrigerator for subsequent experimental detection. 2,3,5-triphenyltetrazolium chloride (TTC) staining was used to measure the cerebral infarction area in mice.

Experiment 2: The mice were divided into four groups: sham, MCAO, MCAO + sh-NC and MCAO + sh-TMED10 (sh-1). A total of 2 $\mu$l of concentrated lentivirus ($1.0 \times 10^9$ TU/ml) was injected into the ischemic lateral ventricles (anteroposterior-0.3 mm, mediolateral-1.0 mm, dorsoventral-2.2 mm to the bregma) of mice. MCAO mouse model were established one week after recovery, and reperfusion was performed after 60 min of occlusion in external carotid artery. Part of mice was participated in histological and molecular tests at this point. And the day was recorded as day 0 of MCAO modeling.

## Morris water maze test

Morris water maze test was performed to test the spatial learning and memory ability of mice. The details of this experiment referred to previous studies [28, 29]. The learning trial was conducted from the day 22 to day 27 of MCAO modeling. The escape latency was recorded during this period. On the day 28, probe test was performed, and platform crossing times of mice were recorded.

## Real-time PCR

Total RNA was isolated from brain tissues and primary cortical neurons using TRIpure lyase (Bioteke, Beijing, China). The RNA samples obtained were reverse transcribed into corresponding cDNA using BeyoRT II M-MLV reverse transcriptase (Beyotime, Shanghai, China) according to the manufacturer's instructions. SYBR Green (Solarbio, Beijing, China) and 2 × Taq PCR Master Mix (Solarbio) were used to detect relative mRNA expression. The primer sequence of TMED10 is as follows: TMED10 F 5′-TCTGTATGCCAAAGAGGATG-3′, TMED10 R 5′-CAATGGACTCGGAAAGGT-3′. The relative mRNA expression was standardized by β-actin, F 5′-CTGTGCCCATCTACGAGGGCTAT-3′, R 5′-TTTGATGTCACGCACGATTTCC-3′.

## Western blot

Proteins were extracted by RIPA lysis buffer (Beyotime) and phenylmethanesulfonyl fluoride (PMSF) (Beyotime). After the protein concentration was measured using BCA Protein Assay Kit (Beyotime), equal amounts of protein were separated by SDS-PAGE. Proteins were then transferred onto the polyvinylidene fluoride (PVDF) membranes (Thermo Fisher Scientific, Pittsburgh, PA, USA). The membranes were incubated with primary antibodies at 4°C overnight after blocking for 1 h. Next, they were incubated with the secondary antibodies labeled horse radish peroxidase (HRP). The details of antibodies were listed in Table1.

## CCK8 assay

The primary cortical neurons were seeded in 96-well culture plates with $4 \times 10^3$ cells in each well. After virus infection for 72 h, the cells were cultured in the environment of oxygen and sugar deprivation for 1 h and then cultured in normal environment for 24 h. 10 $\mu$l CCK8 (Beyotime) solution was added in each well and cultured for 2 h. And then the OD value was measured at 450 nm on a microplate.

## Immunofluorescence assay

The brain tissue was embedded in paraffin and prepared into the 5 $\mu$m-thick sections. And immunofluorescence staining was performed using anti-TMED10 (dilution rate 1: 100, Abclonal, Wuhan, China) and anti-NeuN

**Table 1.** The details of antibodies information

| | Name | Dilution rate | Cat No. | Production company |
|---|---|---|---|---|
| Primary antibodies | TMED10 | 1:500 | A18090 | ABclonal |
| | Cytochrome C | 1:500 | A4912 | ABclonal |
| | p-JNK$^{Thr183/Tyr185}$ | 1:1,000 | AF3318 | Affinity |
| | JNK | 1:500 | AF6318 | Affinity |
| | p-c-Jun$^{Ser73}$ | 1:1,000 | AF3095 | Affinity |
| | c-Jun | 1:500 | AF6090 | Affinity |
| | COX IV | 1:500 | A11631 | ABclonal |
| | β-actin | 1:2,000 | 60008-1-Ig | Proteintech |
| Secondary antibodies | Goat anti-rabbit IgG | 1:10,000 | SA00001-2 | Proteintech |
| | Goat anti-mouse IgG | 1:10,000 | SA00001-1 | Proteintech |

TMED10; transmembrane p24 trafficking protein 10, JNK; c-Jun N-terminal kinase.

(dilution rate 1:200, Abcam, Cambridge, UK) at 4°C overnight. After washing by PBS, cells were incubated in the secondary antibodies FITC labeled goat anti-rabbit IgG (dilution rate 1:200, Abcam) and CY3 labeled goat anti-mouse IgG (dilution rate 1:200, Invitrogen, Carlsbad, CA, USA) at room temperature for 90 min. The nuclei were then stained with DAPI (Aladdin, Shanghai, China). Finally, after cells were covered by the anti-fluorescence quenching agent (Solarbio), the photos of cells were taken under a microscope.

## TUNEL staining

The primary neurons were treated by 200 $\mu$l 0.1% Triton X-100 (Beyotime) with 0.1% sodium citrate at room temperature for 15 min. The TUNEL reaction solution was prepared by the enzyme solution and label solution in the In Situ Cell Death Detection Kit (Red) (Roche, Basel, Switzerland) according to 1:9, then added into the cell plate and incubated at 37°C for 60 min in the dark environment. Cells were photographed under a microscope after the nuclei was stained with DAPI (Aladdin).

TUNEL combined with IF was used to detect neuronal apoptosis. Neurons are located by the primary antibody NeuN (dilution rate 1:200, Abcam) and secondary antibody CY3 labeled goat anti-mouse IgG (dilution rate 1:200, Invitrogen). In Situ Cell Death Detection Kit (Green) (Roche) was used to detect apoptosis.

## ROS test

Intracellular ROS production was determined using a ROS detection kit (KeyGen Biotech., Nanjing, China). The 2, 7-dichlorofluorescein diacetate (DCFH-DA) was diluted with serum-free medium at 1:1,000 to reach a final concentration of 10 $\mu$M. After the cell culture medium was removed, the cells were incubated in the appropriate volume of diluted DCFH-DA at 37°C for 20 min. After washing by the medium, they were photographed under the fluorescence microscope. Reactive oxygen species assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was also used in the examination of ROS production in ischemic penumbra of brain tissue. The tissues were prepared as single cell suspensions containing $1.0 \times 10^6$ or more cells in total. Diluted DCFH-DA (10 $\mu$M) was added in cell solution, and the mixture was incubated at 37°C for 30 min. After cell solution was centrifuged and resuspended in PBS, the fluorescence intensity was detected by a fluorescence microplate reader with excitation wavelength at 488 nm and emission wavelength at 525 nm.

## Mitochondrial membrane potential detection

According to the manufacturer's instructions of mitochondrial membrane potential assay kit with JC-1 (Beyotime), the cells were incubated in 1 ml JC-1 staining solution for 20 min. After washing by the precooled JC-1 staining buffer, the cells were observed under a fluorescence microscope.

## Nissl staining

Tissue samples were embedded in paraffin and sectioned for dewaxing. Each section was dropped with 0.5% cresol violet solution (Sinopharm, Shanghai, China) until the whole tissue was covered, and stained at room temperature for 10 min. The slices were then placed in 0.25% glacial acetic acid ethanol solution (Kemiou Chemical Reagent, Tianjin, China) for several seconds. After the sections were dehydrated and transparent with anhydrous ethanol and xylene, the staining effect was observed under a microscope.

## Statistical analysis

All statistical analysis were performed using GraphPad Prism 8.0. The difference in this study was analyzed by student's *t* test or one way analysis of variance (ANOVA). Data were presented as mean ± SD. $P<0.05$ means significant difference.

## Results

### TMED10 was highly expressed in the cerebral ischemic penumbra of MCAO mouse model

In order to better explore the role of TMED10 in ischemic stroke, we firstly examined its expression in the MCAO/reperfusion mouse model. TTC staining results reflected the situation of cerebral infarction in mice. Compared with the sham mice, the cerebral infarction was observed in the cerebral ischemic penumbra of MCAO mouse model (Fig. 1A), which revealed that the MCAO model was successfully established to simulate ischemic stroke. As shown in Fig. 1B, the mRNA and protein expression of TMED10 was obviously increased in the cerebral ischemic penumbra of MCAO mice compared with the sham group. Besides, IF results exhibited that the cerebral ischemic penumbra was successfully stained by NeuN, and the expression of TMED10 in cortical neurons of MCAO mice was significantly higher than that of sham mice (Fig. 1C). These findings suggested that TMED10 was highly expressed in the cerebral ischemic penumbra of MCAO mice model.

### TMED10 protected neurons from apoptosis induced by OGD/R

The primary cortical neurons were isolated from the cerebral cortex tissues of newborn C57BL/6J mice (Supplementary Fig. 1). The mRNA and protein expression of TMED10 in cortical neurons were suppressed by sh-1 and sh-2 (Fig. 2A) and overexpressed by OE-TMED10 (Supplementary Fig. 2A). Under OGD/R condition, TMED10 expression was significantly increased at the transcription and translation levels, and it was significantly inhibited by sh-1 and sh-2 (Fig. 2B), and was upregulated by OE-TMED10 (Supplementary Fig. 2B). CCK8 results displayed that cell viability was notably blocked in the neurons exposed to OGD/R. The knockdown of TMED10 further inhibited cellular activity (Fig. 2C). Besides, the apoptosis of neurons was induced by OGD/R treatment. TUNEL-positive rate of neurons was obviously reduced by OE-TMED10 (Supplementary Fig. 2D), and was significantly raised by sh-1and sh-2 (Fig. 2D). Therefore, the results above demonstrated that the silence of TMED10 suppressed cell survival and aggravated neuron apoptosis induced by OGD/R. In a word, TMED10 could protect neurons from OGD/R-induced cell apoptosis.

### Reduction of TMED10 promoted the mitochondrial injury of OGD/R-induced neurons

It is known that in the hypoxia and ischemia environment, excessive ROS production can lead to mitochondrial damage, which promotes cell necrosis and apoptosis in turn [3]. Meanwhile, impaired mitochondrial may

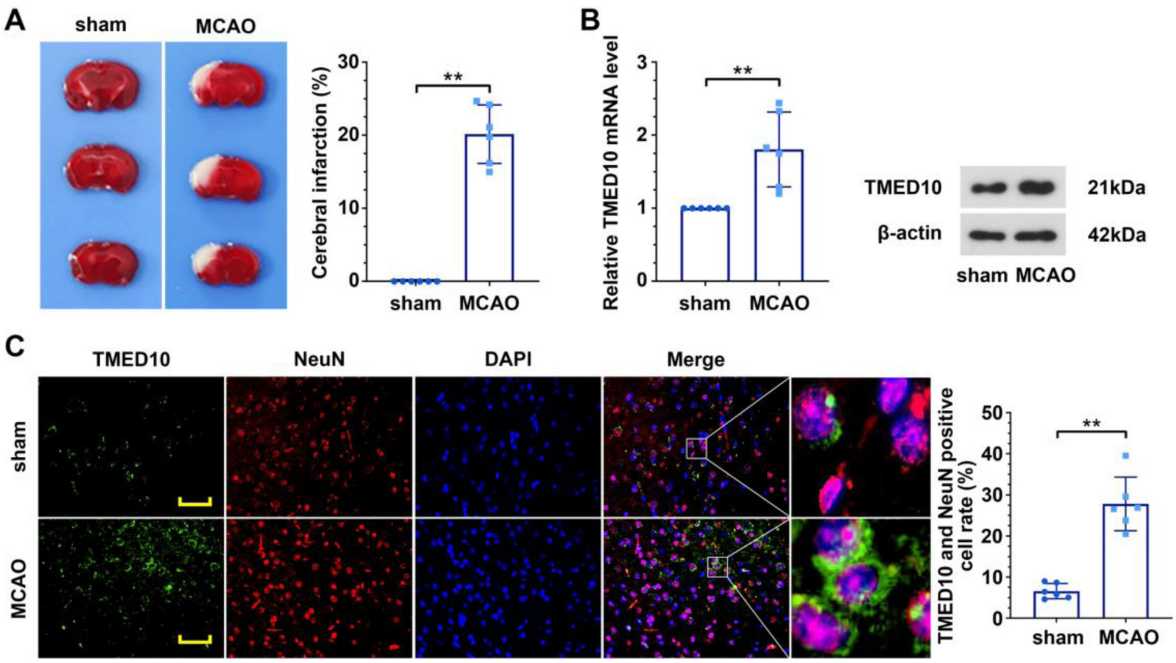


**Fig. 1.** Transmembrane p24 trafficking protein 10 (TMED10) highly expressed in the cerebral ischemic penumbra of MCAO (middle cerebral artery occlusion) mice. A. 2,3,5-triphenyltetrazolium chloride (TTC) staining detected the cerebral infarction in the cerebral ischemic penumbra of MCAO mice. B. Real-time PCR and western blot examined the mRNA and protein expression of TMED10. C. The TMED10-NeuN immunofluorescence double staining evaluated the expression of TMED10 in mouse cortical neurons. Scale bar=50 $\mu$m. Results came from six independent replicates. Data presented are mean ± SD. $^{**}P<0.01$.

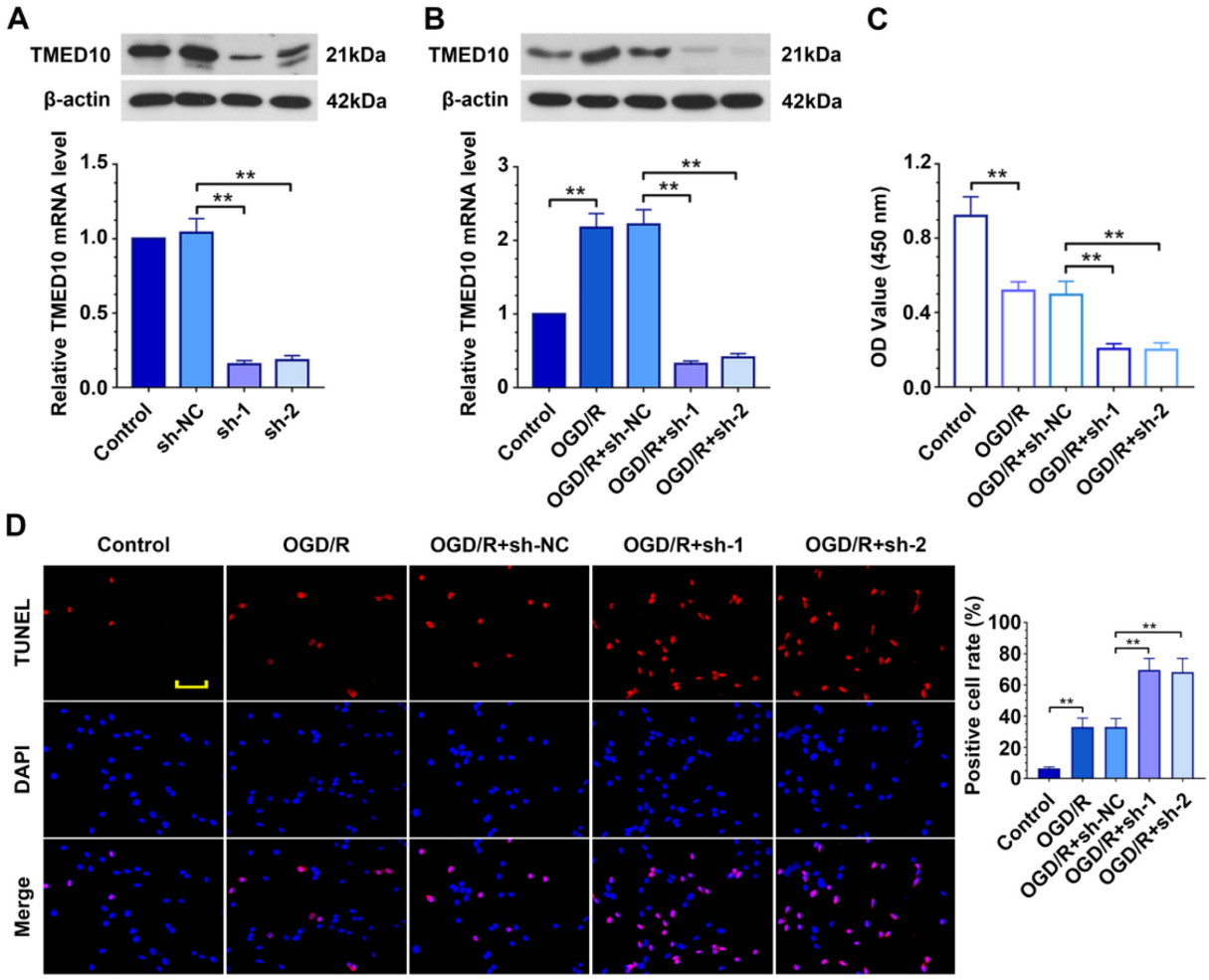


**Fig. 2.** The effect of transmembrane p24 trafficking protein 10 (TMED10) on neuronal apoptosis induced by oxygen glucose deprivation/reoxygenation (OGD/R). A. Real-time PCR and western blot detected the efficiency of sh-TMED10-1 (sh-1), sh-TMED10-2 (sh-2) at mRNA level and protein level. B. Real-time PCR and western blot examined the TMED10 expression in neurons exposed to OGD/R. C. CCK8 assay evaluated cell viability. D. Apoptosis was tested in neurons after TMED10 silence by TUNEL staining. Scale bar=50 $\mu$m. Results came from three independent replicates. Data presented are mean ± SD. ***P*<0.01.

result in the oxidative stress that affects the normal biological behavior of cells [11]. The fluorescence intensity in Fig. 3A reflects the production of ROS. ROS production was obviously increased in neurons under OGD/R condition, and was further improved by TMED10 reduction. Cytochrome c, which participates in the mitochondria ATP synthesis, triggers programmed cell death after released into the cytoplasm [30]. The protein expression of cytochrome c in the cytoplasm was raised up by OGD/R treatment and was further upregulated by shTMED10s. Additionally, the cytochrome c expression in the mitochondria was obviously reduced in the neurons exposed to OGD/R and was further downregulated by shTMED10s (Fig. 3B). The changes of mitochondrial membrane potential were verified by JC-1 staining. In normal mitochondria, JC-1 dye makes the mitochondrial matrix fluoresce red. The damaged mitochondrial membrane potential will decrease or lose, and green fluorescence will be produced at this time. The ratio of red fluorescence to green fluorescence represents the degree of mitochondrial damage. In Fig. 3C, the suppression of TMED10 significantly declined the ratio of red to green fluorescence under OGD/R condition. The oxidative damage of mitochondria was induced by the downregulation of TMED10. These results indicated that TMED10 as a cell protector suppressed the excessive production of ROS and mitochondrial damage of neurons induced by OGD/R.

## The JNK pathway mediated the aggravating effect of TMED10 reduction on neuronal mitochondrial damage

The JNK pathway has been verified to play a crucial role in mitochondrial damage-induced apoptosis [12]. However, it remains to be explored whether this signaling pathway is involved in the regulation of TMED10

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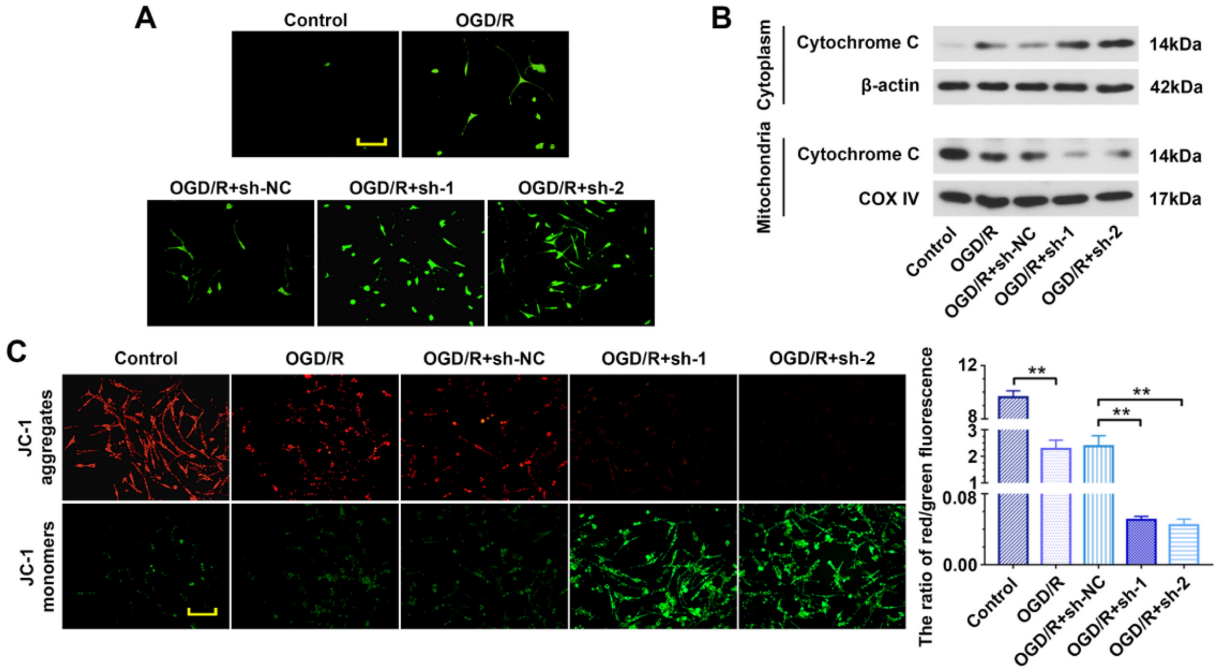


**Fig. 3.** Transmembrane p24 trafficking protein 10 (TMED10) suppression promoted the mitochondria damage induced by oxygen glucose deprivation/reoxygenation (OGD/R). A. Reactive oxygen species (ROS) production in cells was detected by the ROS detection kit. Scale bar=100 $\mu$m. B. The protein expression of cytochrome c in cytoplasm and mitochondria was detected by western blot. C. The changes of mitochondrial membrane potential were verified by JC-1 staining. Scale bar=100 $\mu$m. Results came from three independent replicates. Data presented are mean ± SD. $^{**}P<0.01$.

on neuron viability and apoptosis. Western blot results in Fig. 4A displayed that the expression of activated JNK (p-JNK$^{Thr183/Tyr185}$) and activated c-Jun (p-c-Jun$^{Ser73}$) was raised by OGD/R and further increased through reducing TMED10. In addition, the expression of JNK and c-Jun did not change markedly during the treatment of OGD/R and sh-1/sh-2. The activation of JNK signaling has been reported to induce by the environmental stress like OGD/R condition [31, 32] and promotes the expression of transcription factor c-Jun [12], which could induce the neuronal death through mitochondrial injury directly [33]. In addition, compared with the OGD/R and sh-1/sh-2 group, cytochrome c was downregulated in cytoplasm and was upregulated in mitochondria after neurons were treated by SP600125, which was the inhibitor of JNK pathway. The sh-TMED10s induced release of cytochrome c from mitochondria was blocked by SP600125 (Fig. 4B). These results indicated that TMED10 knockdown induced mitochondrial damage was rescued by blocking the JNK signaling pathway. As shown in Fig. 4C, results of JC-1 dye presented that the ratio of red to green fluorescence was significantly decreased by sh-1/sh-2 and was rescued by the JNK inhibitor SP600125. TUNEL staining indicated that neuron apoptosis was significantly promoted through silencing TMED10, while it was remarkably suppressed with the addition of SP600125 (Fig. 4D). Moreover, in the neurons exposed to OGD/R, the expression of p-JNK$^{Thr183/Tyr185}$ and p-c-Jun$^{Ser73}$ was obviously downregulated when TMED10 was overexpressed. Meanwhile, JNK and c-Jun hardly changed in the neurons with OE-TMED10 under OGD/R condition (Supplement Fig. 2C). The above findings verified that the JNK signaling pathway mediated the effect of TMED10 on mitochondrial oxidative damage and apoptosis in neurons.

## Knockdown of TMED10 aggravated the MCAO-induced brain damage

To investigate the effect of TMED10 on MCAO-induced brain injury, cerebral infarction size was measured in the cerebral ischemic penumbra of MCAO mice by TTC. As depict in Fig. 5A, the protein expression of TMED10 was increased in the cerebral ischemic penumbra of MCAO mice and was obviously reduced by sh-1. The cerebral infarction was appeared in the brain tissue of MCAO mice and was further raised by sh-1 (Fig. 5B). The results of Nissl staining suggested that the damaged neurons in MCAO mice was more than that in sham mice. Meanwhile, the neuronal injury was exacerbated by sh-1 (Fig. 5C). The DCFH-DA fluorescence intensity is positively related to the ROS production. So, the ROS production was upregulated in the cerebral ischemic penumbra of MCAO mice and was further promoted by sh-1 (Fig. 5D). Besides, the cytochrome c

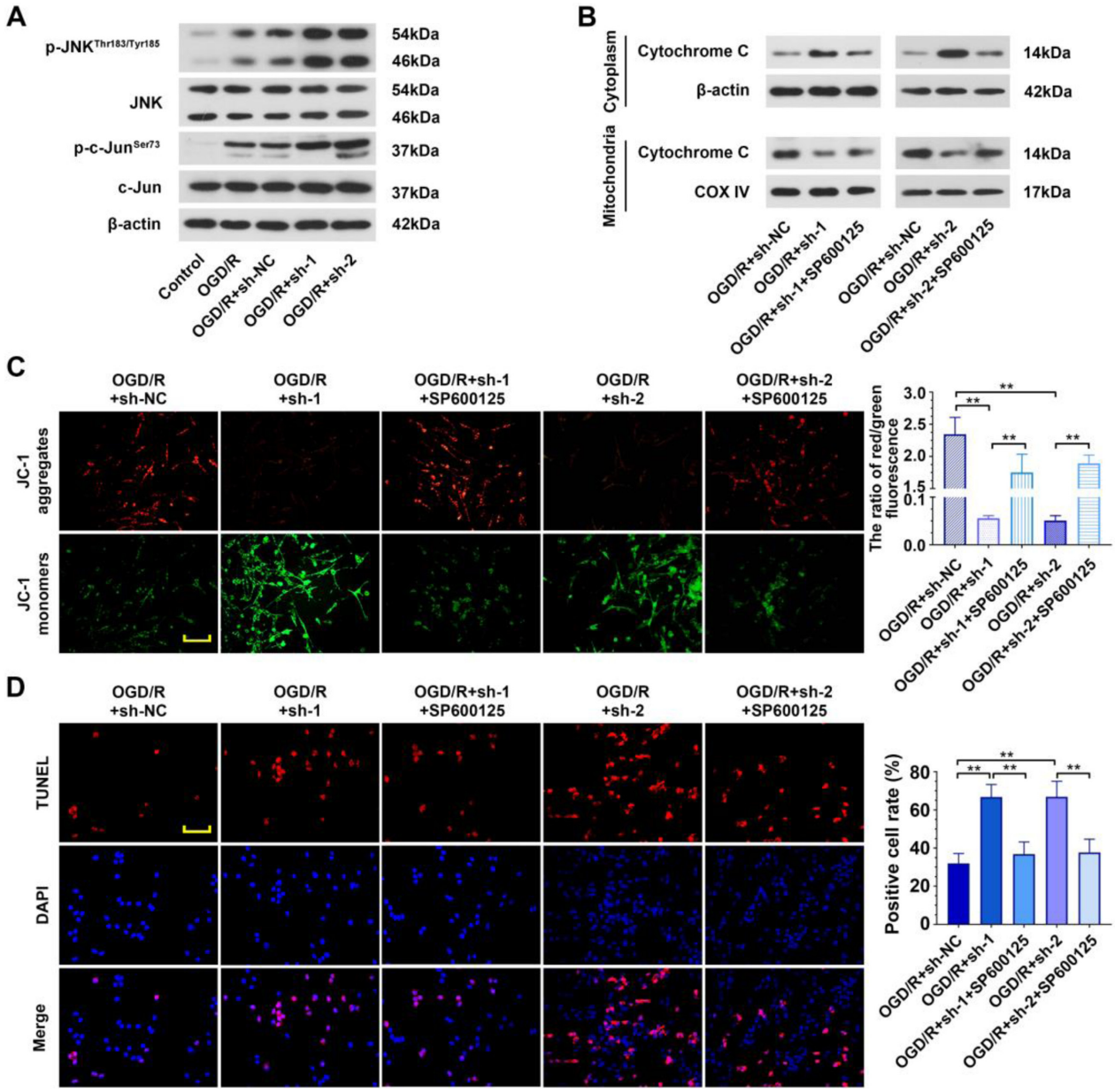


**Fig. 4.** The c-Jun N-terminal kinase (JNK) pathway mediated the exacerbation of neuronal apoptosis and mitochondria damage induced by transmembrane p24 trafficking protein 10 (TMED10) downregulation. A. The expressions of p-JNK$^{Thr183/Tyr185}$, JNK, p-c-Jun$^{Ser73}$ and c-Jun were detected by western blot. B. Cytochrome c expression in cytoplasm and mitochondria of neurons affected by sh-1/sh-2 and SP600125 was detected by western blot. C. The mitochondrial membrane potential of neurons was examined by JC-1 staining. Scale bar=100 $\mu$m. D. Cell apoptosis was tested by TUNEL staining. Scale bar=50 $\mu$m. Results came from three independent replicates. Data presented are mean ± SD. $^{**}P<0.01$.

expressed in cytoplasm was further promoted by sh-1 in the cerebral ischemic penumbra of MCAO mice. While, the cytochrome c was obviously suppressed in the neuronal mitochondria and was further decreased by sh-1 (Fig. 6A). As shown in Fig. 6B, the phosphorylated JNK and c-Jun were enhanced by the sh-1 in the cerebral ischemic penumbra of MCAO mice. The results verified that sh-1 induced cerebral ischemic was mediated by the JNK pathway *in vivo*. Moreover, TUNEL positive neurons were increased in the cerebral ischemic penumbra of MCAO mice. When TMED10 was downregulated, neurons apoptosis was further promoted (Fig. 6C). The results demonstrated that TMED10 downregulation exacerbated the brain damage via activating the JNK pathway. Taken together, TMED10 protected neurons from cerebral ischemia through blocking the JNK signaling pathway.

## Silence of TMED10 promoted MCAO-induced cognitive impairment in mice

Morris water maze experiment was performed to explore the effect of TMED10 on the cognitive impairment.

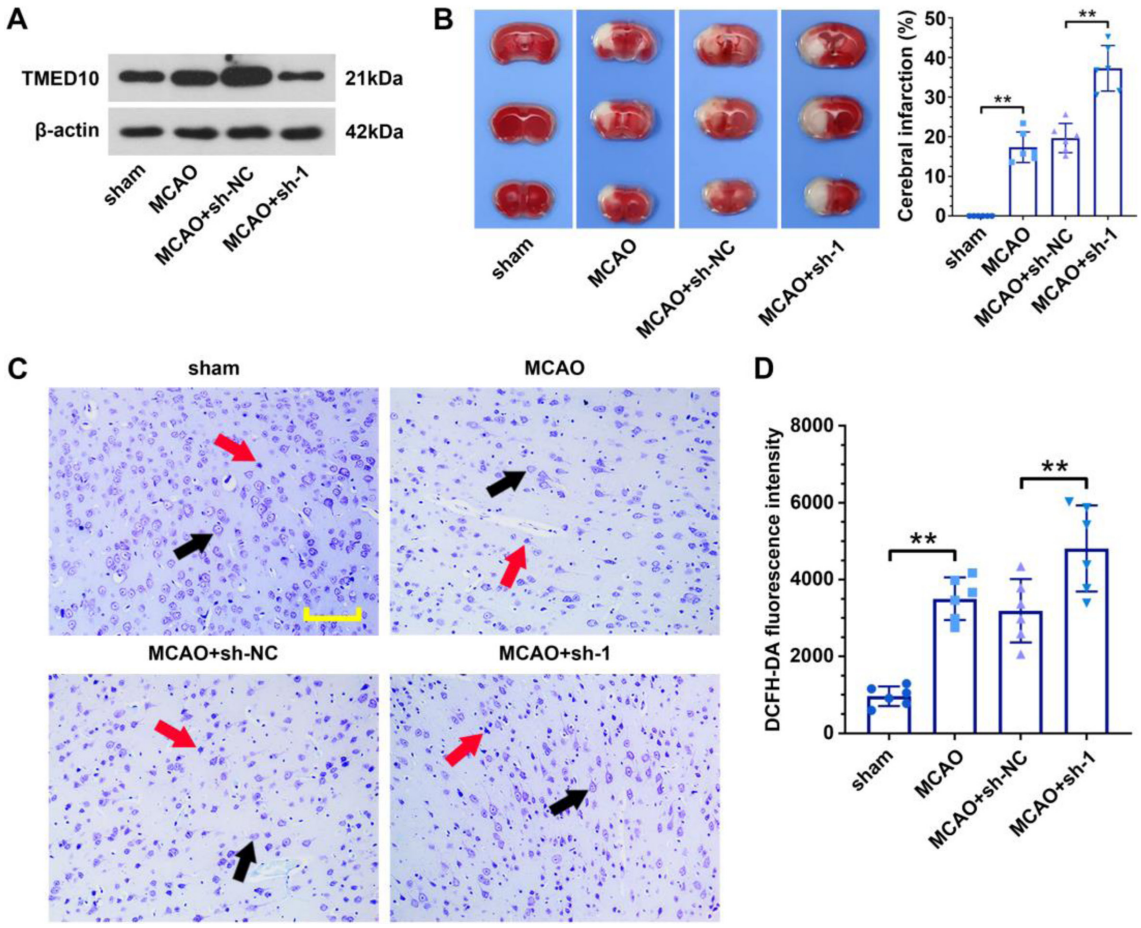


**Fig. 5.** Transmembrane p24 trafficking protein 10 (TMED10) reduction aggravated cerebral infarction and neuronal injury induced by middle cerebral artery occlusion (MCAO). A. The protein expression of TMED10 in the cerebral ischemic penumbra of MCAO mice was measured by western blot. B. 2,3,5-triphenyltetrazolium chloride (TTC) staining detected the cerebral infarction area in the cerebral ischemic penumbra. C. Nissl staining evaluated the neuronal injury in penumbra area of ischemic cortex. Black arrows mark the normal neurons. Red arrows mark the damaged neurons. Scale bar=100 $\mu$m. D. The 2, 7-dichlorofluorescein diacetate (DCFH-DA) fluorescence intensity represents the reactive oxygen species (ROS) production. Results came from six independent replicates. Data presented are mean ± SD. $^{**}P<0.01$.

The procedure of this experiment was illustrated in Fig. 7A. The escape latency during learning trail on different treatment demonstrated that the memory ability of MCAO mice was impaired compared with sham mice. The cognitive impairment of MCAO mice induced by sh-1 was more severe than the control mice (Fig. 7B). The movement track of mice on day28 was also recorded as shown in Fig. 7C. We recorded the times of experimental mice crossed the platform as depict in Fig. 7D. Compared with sham mice, the number of times that MCAO mice crossed the target location of platform were significantly declined. When TMED10 expression was inhibited, the platform crossing times was lower than the control mice. Therefore, we verified that the down-regulation of TMED10 would exacerbate the cognitive impairment induced by the MCAO treatment.

## Discussion

In the current study, we found that TMED10 expression was upregulated after OGD/R treatment. The purpose of this study was to investigate the effect of TMED10 on cerebral stroke caused by ischemic and hypoxia. For the above reasons, we silenced TMED10 in neurons under OGD/R condition, and suppressed TMED10 in the cerebral ischemic penumbra of MCAO mice. We verified the effect of TMED10 on cerebral infarction or nerve cell injury and death in a MCAO-induced ischemic stroke mouse model, and examined the mechanism of TMED10 on regulating the mitochondrial injury and apoptosis in primary cortical nerve cells induced by OGD/R.

Stroke is a cerebrovascular disease with high mortality rate all over the world [34]. The probability of its occurrence is increasing as the population aging [35].

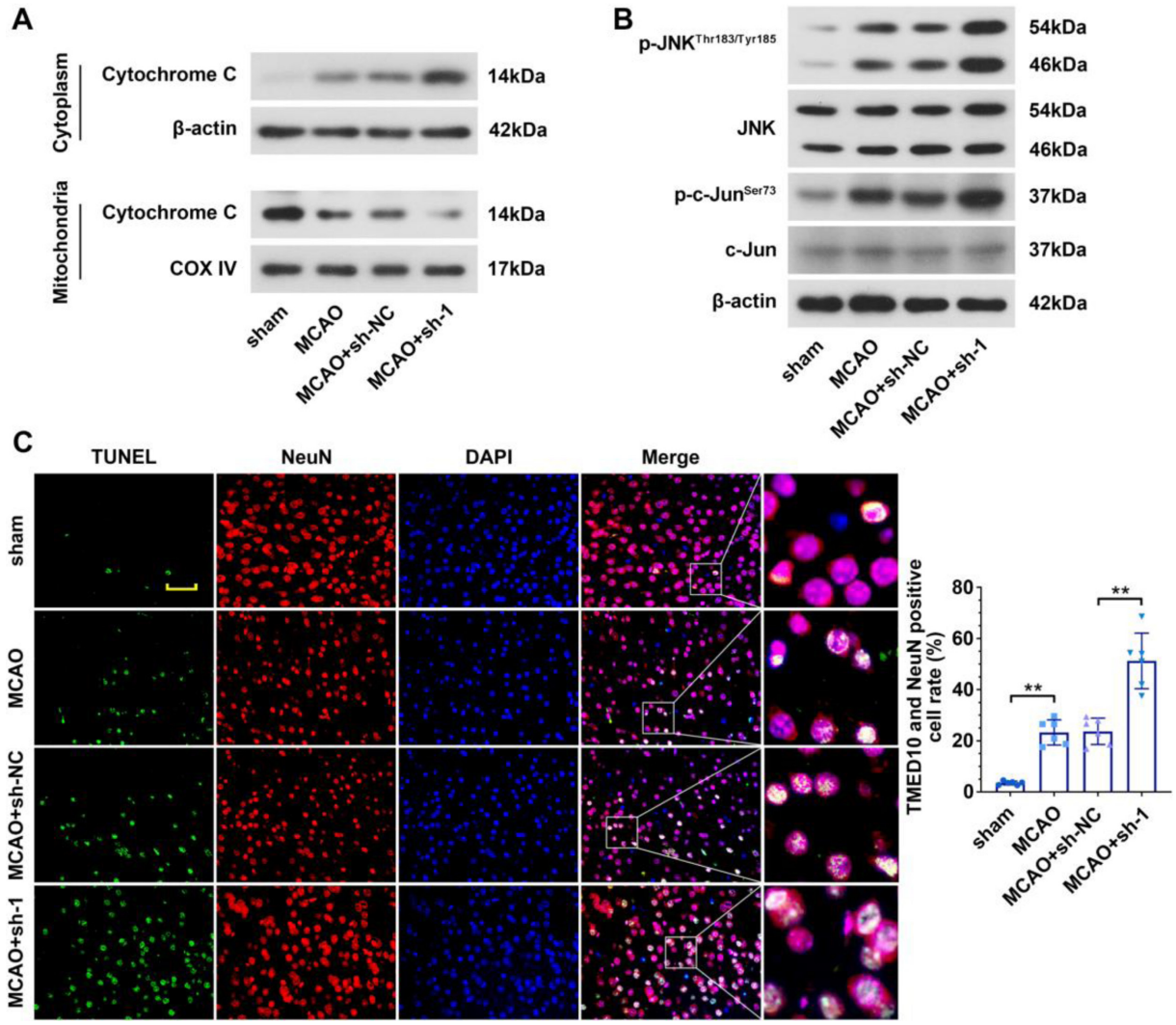


**Fig. 6.** The decrease of transmembrane p24 trafficking protein 10 (TMED10) induced neuronal apoptosis was mediated by the c-Jun N-terminal kinase (JNK) pathway. A. Cytochrome c expressed in cytoplasm and mitochondria was evaluated by western blot. B. The expressions of p-JNK$^{Thr183/Tyr185}$, JNK, p-c-Jun$^{Ser73}$ and c-Jun were detected by western blot. C. TUNEL-NeuN fluorescence double staining was used to detect neuronal apoptosis in the ischemic penumbra area of brain cortex. Scale bar=50 $\mu$m. Results came from six independent replicates. Data presented are mean ± SD. $^{**}P<0.01$.

Stroke is classified as ischemic stroke caused by sudden blockage of an artery and hemorrhagic stroke caused by rupture of a blood vessel and bleeding of brain tissue [36]. Cognitive impairment is a serious sequelae associated with stroke survivors [37, 38]. At present, thrombolysis or endovascular therapy can be used for ischemic stroke [39], but the clinical application of these methods is limited. Studies have shown that the axis of tyrosine kinase receptor c-Met and hepatocyte growth factor (HGF) as the anti-apoptotic factors to protect neurons in a MCAO rat model through activating the STAT3 signaling pathway [40]. Besides, a chemical molecule named JLX001 was found to protect the neuronal injury via blocking the JNK signaling pathway [12]. In addition, vitexin presented in some plants was shown to attenuate the cerebral ischemic stroke through the mTOR/Ulk1 pathway in MCAO rats [41]. Therefore, the current study aims to explore molecular therapeutic targets to ameliorate ischemic stroke and cognitive impairment. We successfully established the MCAO mouse model, which is the closest animal model to human ischemic stroke [12]. TMED10 was found to highly expressed in the cerebral ischemic penumbra of MCAO mice. And its downregulation induced a further increase of the cerebral infarction area in the brain tissue of MCAO mice. Meanwhile, the neuronal apoptosis was exacerbated by the suppression of TMED10 and was inhibited through blocking the JNK signaling pathway. To sum up, TMED10 could protect neurons from MCAO-induced cell apoptosis through the JNK signaling pathway. The study provides a potential molecular target for ischemic stroke and cognitive impairment.

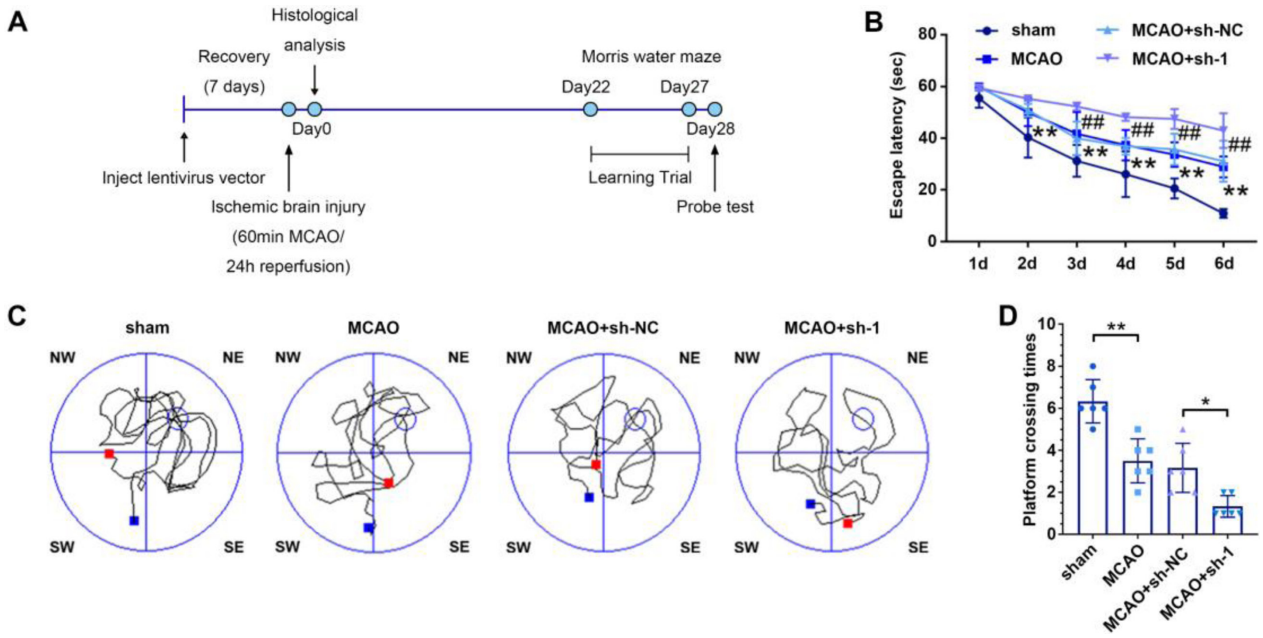


**Fig. 7.** Downregulation of transmembrane p24 trafficking protein 10 (TMED10) aggravated the cognitive impairment induced by middle cerebral artery occlusion (MCAO). A. The time axis of mouse cognitive experiment containing MCAO modeling and Morris water maze. B. Escape latency of spatial learning during memory ability training. $^{**}P<0.01$ sham vs MCAO, $^{\#\#}P<0.01$ MCAO + sh-NC vs MCAO + sh-1. C. Movement trajectory of mice during probe test on day 28. The circle in northeast (NE) area was the location of platforms during learning trial. D. Platform crossing times of mice during probe test. Results came from six independent replicates. Data presented are mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$.

We already know that the human brain accounts for about 2% of a person's body weight, yet it consumes about 20% of the body's total oxygen consumption [42]. Neurons, as important functional units in the brain, have a higher oxygen demand and metabolic rate, so they are more vulnerable to oxidative damage [43]. The basic mechanism of cell damage after cerebral ischemia is oxidative stress caused by ROS overproduction [44], which cause mitochondrial respiratory chain enzyme damage at the same time [8]. In our research, OGD/R treatment simulated ischemic stroke upregulated ROS production and decreased mitochondrial membrane potential in neurons. This further confirms that ischemic stroke results in oxidative stress and mitochondrial damage in nerve cells. Several researches have verified that excessive ROS production induced by the mitochondrial defects promotes the neurodegenerative disease, such as cognitive impairment [11]. Based on these studies, the production of ROS and the changes of mitochondrial membrane potential were examined in the neurons under OGD/R condition. Thus, with the decrease of TMED10 expression, the ROS production was raised up, the release of cytochrome c was promoted, and the mitochondrial membrane potential dropped sharply. The aggravation of oxidative stress and mitochondrial damage in neurons caused by sh-1 further leads to inhibition of cell survival and promotion of cell apoptosis. These results provide important evidence for TMED10 as a potential therapeutic target for ischemic stroke.

According to several studies, the JNK signaling pathway is involved in the regulation of various physiological or pathological processes of the nervous system, mediating the death of nerve cells due to stress or injury [45]. The JNK family is thought to participate in the stress-induced apoptosis and nervous system damage, as well as neuropsychiatric disorders and other pathological processes [46–48]. Previous studies have suggested that JNK signaling pathway is involved in neuronal apoptosis induced by cerebral ischemia [49], and could be activated by ischemia and reperfusion [50]. In addition, one study has found that the upregulation of activated JNK results in an increase in TUNEL-positive neurons during focal cerebral ischemia, suggesting that inhibition of the JNK pathway may be a potential approach to protect ischemic brain tissue [51]. Besides, several studies demonstrated that the activation of JNK pathway could exacerbate mitochondrial damage and accompanied ROS production [11, 12], further, the excessive ROS could in turn activate the JNK pathway [52, 53]. Thus, we speculate that activation of JNK pathway and ROS production promote each other to exacerbate neuronal damage during ischemic stroke. Moreover, the present study indicated that the JNK and c-Jun were activated by OGD/R or MCAO. With TMED10 down-

regulation, the phosphorylated JNK and c-Jun were further facilitated in the neurons of cerebral ischemic penumbra. The JNK inhibitor SP600125, also as a neuroprotectants, rescued the neuron apoptosis induced by the downregulation of TMED10. In other words, the protective effect of TMED10 on neuronal injury induced by cerebral ischemia was mediated the JNK pathway.

In conclusion, TMED10 is found to highly expressed in ischemic stroke models. Neuronal apoptosis is promoted by the excessive ROS production and mitochondrial damage induced by cerebral ischemia. TMED10 at least partly protects neurons from oxidative stress and mitochondrial injury through the JNK signaling pathway. TMED10 may be a potential therapeutic target for cognitive impairment caused by ischemic stroke.

## Acknowledgment

Not applicable.

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