Tubeimoside I ameliorates cerebral ischemia/reperfusion injury through activating SIRT3

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Abstract

Cerebral ischemia-reperfusion (CIR) seriously affects human health and life as it is accompanied by inflammation and apoptosis in brain tissues. Tubeimoside I (TBMS-1) can inhibit neuroinflammation and has neuroprotective effects; however, its effects on ischemia-reperfusion (IR) injury of the brain requires clarity. A mouse cerebral artery occlusion/reperfusion model was used to simulate CIR injury. The neurological function and the area of cerebral infarction were assessed by 2,3,5-triphenyltetrazolium chloride staining. Tumor necrosis factor-α, Interleukin (IL)-1β, IL-6, and IL-10 levels were measured by enzyme-linked-immunosorbent serologic assay kits. Protein blot analysis was performed to assess the expression of apoptosis-related factors. In addition, PC12 (pheochromocytoma) cells were treated with oxygen–glucose deprivation/reoxygenation (OGD/R) to establish an *in vitro* model of CIR injury. The cell viability was measured by cell counting kit-8 assay, and apoptosis levels were detected by flow cytometry. *In vivo* results indicated that Tubeimoside I reduced cerebral infarct size, decreased inflammatory factor content, inhibited the expression of apoptosis-related factors, including Bax and cleaved-caspase-3 (Asp175), and promoted the expression of survival factor, such as B-cell lymphoma protein *2*. *In vitro*, Tubeimoside I was able to increase cell viability and inhibit apoptosis. Mechanistically, Tubeimoside I was able to enhance both *in vivo* and *in vitro* expressions of NAD-dependent deacetylase sirtuin-3 (SIRT3). SIRT3 inhibitor abolished the protective effect of Tubeimoside I on OGD/R-treated cells. Tubeimoside I lessened CIR injury by activating SIRT3. Hence, it could be a potential drug candidate for treating IR injury of the brain.

Keywords: cerebral ischemia-reperfusion (CIR); Tubeimoside I; inflammation; apoptosis; SIRT3

Introduction

Stroke is a cerebrovascular disease, with approximately more than 80% strokes identified as ischemic strokes (Yao *et al.*, 2021). Moreover, ischemic stroke is the second leading cause of death among human illnesses (Zeng *et al.*, 2022). Mechanical embolization and intravenous fibrinogen activator are often used to restore blood supply to ischemic brain tissue, but restoring blood flow can also cause brain injury, secondary to brain damage known as cerebral ischemia-reperfusion (CIR), which leads to high mortality and disability proportions. The development of CIR is usually accompanied by inflammation and oxidative stress, which ultimately leads to apoptosis and necrosis of ischemic brain tissue (Yang *et al.*, 2021b). Presently, much of the research is devoted to finding anti-inflammatory and neuroprotective agents that can lessen CIR damage. The protective effect of Chinese medicine on CIR injury is receiving increasing attention.

Tubeimoside I, also known as Tubeimoside A (TBMS-1), is a naturally occurring substance that is obtained from the plant *Bolbostemma paniculatum*. It is a traditional Chinese herbal remedy that has been used for centuries to cure a variety of illnesses (Du *et al.*, 2020). In recently conducted studies, Tubeimoside I has demonstrated prominent anti-inflammatory and antioxidant properties. For instance, Tubeimoside I inhibited the production of inflammatory cytokines in rat arthritis (Liu *et al.*, 2018), attenuated particulate matter 2.5 (PM 2.5)-induced lung inflammation and oxidative damage (Zhang *et al.*, 2018), and prevented microglia-mediated inflammation (He *et al.*, 2018). In addition, Tubeimoside I has been shown to lessen oxidative stress, infarct size, and apoptosis, thereby reducing myocardial ischemia-reperfusion injury by activating NAD-dependent deacetylase sirtuin-3 protein (SIRT3) (Lv *et al.*, 2021). However, the role of Tubeimoside I in CIR requires clarity.

In this study, we discovered that Tubeimoside I ameliorated brain infarction caused by CIR injury *in vivo* and inhibited the expression of inflammation, oxidative stress, and apoptosis-related factors. Tubeimoside I ameliorated *in vitro* oxygen–glucose deprivation/reoxygenation (OGD/R)-induced apoptosis, but was reversed by SIRT3 inhibitors. This suggested that Tubeimoside I ameliorated CIR injury by activating SIRT3 and could be a possible medication for treating CIR injury.

Methods

Cell culture and treatment

In order to detect the cytotoxicity of different concentrations of Tubeimoside I, rat pheochromocytoma cells PC12 (Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai, China) were cultured in a medium containing different concentrations of Tubeimoside I at 37°C, 95% O_2, and 5% CO_2. To establish an *in vitro* model of CIR, PC12 cells were placed in a glucose-free medium and cultured under hypoxic conditions (1% O_2; 5% CO_2; and 94% N_2); after 3 h of glucose–oxygen deprivation, the complete medium was replaced and the cells were cultured for 24 h at 37°C, 5% CO_2, and 95% O_2. In the Tubeimoside I group, the culture medium was replaced with a medium containing 0.5-μm or 1-μm Tubeimoside I. Cells treated without OGD/R were used as a control group.

Cell counting kit-8 (CCK-8) assay

1×10^4 cells/well were inoculated in a 96-well plate. The cells were treated with different concentrations of Tubeimoside I and incubated for 24 h. Each well received 10 μL of CCK-8 reagent, which was then incubated for 2 h. Absorbance values were measured at 450 nm.

Flow cytometry

After 5-min centrifugation of cells, the supernatant was discarded. Cells were then suspended in 500 μL of 1× binding buffer, and 5 μL of Annexin V-FITC was added. Next, the cells were incubated for 5 min at 37°C protected from light. Then, 5-μL propidium iodide (PI) and 400-μL phosphate-buffered saline (PBS) were added to the cells and incubated for 5 min, and the rate of apoptosis was observed by flow cytometry.

Animals and treatment

Male C57BL/6 mice (6–8-week old) were purchased from Shanghai Jihui Experimental Animal Breeding Co., Shanghai, China. In order to establish arterial occlusion model, ketamine (100 mg/kg) and xylazine (10 mg/kg) were administered to mice to make them unconscious. The left common carotid artery was exposed, and a monofilament nylon suture with a round tip was delicately advanced from external to internal carotid arteries. The carotid artery was occluded for 60 min, and the blood flow was restored (Liu *et al.*, 2020). In the sham group, the monofilament nylon suture was not allowed to enter the internal carotid artery, and mice in the Tubeimoside I group were injected intraperitoneally with 2-mg/kg or 4-mg/kg Tubeimoside I after 60 min of arterial occlusion. According to the National Institutes of Health Laboratory Animal Care and Use Guidelines, animal research was cleared for use by the Cangzhou Central Hospital Ethics Committee.

Neurological function measurement

After 24 h of restoration of blood flow, the neurological function of each group of mice was scored as described by Zhao *et al.* (2020). Depending on the severity, the score was 0–4. After determining neurological function scores, mice were euthanized by cervical dislocation.

Enzyme-linked-immunosorbent serologic assay (ELISA)

The serum of mice was separated by centrifugation, and the levels of tumor necrosis factor-α (TNF-α) (E-EL-M3063; Elabscience, China), interleukin (IL)-1β (E-EL-M0037c; Elabscience), IL-6 (E-EL-M0044c; Elabscience), and IL-10 (E-EL-M0046c; Elabscience) in the serum were evaluated according the manufacturer's guidelines (Yang *et al.*, 2022).

TTC staining

Mouse brain tissues were rapidly frozen and sectioned, and 2% 2,3,5-triphenyltetrazolium chloride (TTC) was added to the sections and incubated for 30 min at 37°C. Subsequently, section photographs were scanned in a computer and measured using image analysis software.

Western-blot analysis

Mouse brain tissues or cells were lysed with radioimmunoprecipitation assay (RIPA) lysis solution, and the proteins were quantified using the bicinchoninic acid (BCA) kit and separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis. The protein bands were transferred to a polyvinylidene difluoride (PVDF) membrane, and closed with 5% skimmed milk powder, washed, and incubated with the corresponding antibodies at 4°C overnight. Next day, the PVDF membrane was washed and incubated with horseradish peroxidase (HPR)-labeled immunoglobulin G (IgG) antibody (1:1,000; GTX213110-01; Genetex US) at room temperature for 2 h. The electrochemiluminescence (ECL) kit was used to allow the strips to develop color, which were placed in a gel imaging system for imaging. Details of all antibodies are as follows: Bax (1:1,000; GTX55531; Genetex), B-cell lymphoma protein *2 (*Bcl-2; 1:1000; GTX01194; Genetex), cleaved-caspase-3 (Asp175, 1:1,000; GTX86952; Genetex), SIRT3 (1:1,000; GTX03711; Genetex), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:5,000; GTX100118; Genetex).

Statistical analysis

GraphPad Prism 5.0 was used for statistical analyses. Data results were expressed as mean ± standard deviation (SD). Comparisons between multiple groups were performed by one-way analysis of variance (ANOVA). For measurements, $P < 0.05$ was considered statistically significant.

Results

Tubeimoside I attenuates OGD/R-induced cell damage

First, we treated PC12 cells with different concentrations of Tubeimoside I (Figure 1A) and found that 0.5-μm and 1-μm Tubeimoside I had no effect on cell activity (Figure 1B). Next, we treated PC12 cells with OGD/R for simulating *in vitro* ischemia/reperfusion injury, and treated the same with 0.5-μm and 1-μM Tubeimoside I. Then, we detected the cell viability by CCK8, and found that 0.5-μM and 1-μM Tubeimoside I could

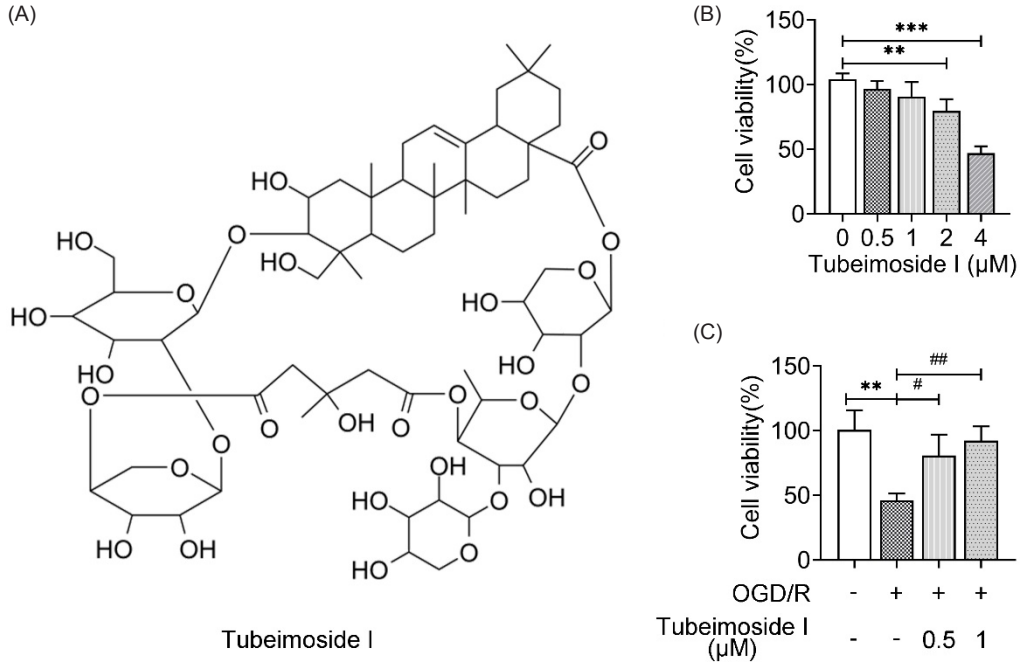

Figure 1. Tubeimoside I attenuates OGD/R-induced cell damage. (A) Tubeimoside I structural formula. (B) Effects of different concentrations of Tubeimoside I on the viability of PC12 cells. (C) Effect of Tubeimoside I on OGD/R-induced cell viability. **P < 0.01, ***P < 0.001; ##P < 0.01 and #P < 0.05 vs OGD/R group.

significantly increase the cell viability induced by OGD/R (Figure 1C).

Tubeimoside I attenuates cerebral infarction in middle cerebral artery occlusion (MCAO) mice

We established a mice MCAO model. Tubeimoside I was injected intraperitoneally 1 h prior to MCAO and 12 h post-MCAO, and neurological function scores were recorded 24 h following MCAO. We observed that MCAO caused a significant increase in neurological function scores, while Tubeimoside I was able to decrease neurological function scores (Figure 2A). We then examined brain infarction in each group of mice by TTC assay and found that the infarct area in MCAO mice was significantly increased whereas the same area in Tubeimoside I group mice was significantly decreased (Figure 2B). These results indicated that Tubeimoside I improved brain infarction.

Tubeimoside I attenuates inflammation and apoptosis in MCAO mice

We examined the serum TNF-α, IL-1β, IL-6, and IL-10 levels in mice by ELISA. We observed that the levels of TNF-α, IL-1β, IL-6, and IL-10 were significantly increased in the MCAO group of mice, while the levels of serum TNF-α, IL-1β, and IL-6 were significantly decreased in MCAO mice treated with Tubeimoside I. However, IL-10 levels were dramatically increased by Tubeimoside I treatment (Figure 3A). We also examined the expression of apoptosis-related factors, including Bax, Bcl-2, and cleaved-caspase-3 by Western-blot analysis, and discovered that the expression of Bax and cleaved-caspase-3 was increased significantly whereas that of Bcl-2 was decreased significantly in the brain tissues of MCAO mice. Tubeimoside I ameliorated the above-mentioned changes (Figure 3B), indicating that Tubeimoside I inhibited the MCAO-induced inflammatory response and tissue apoptosis.

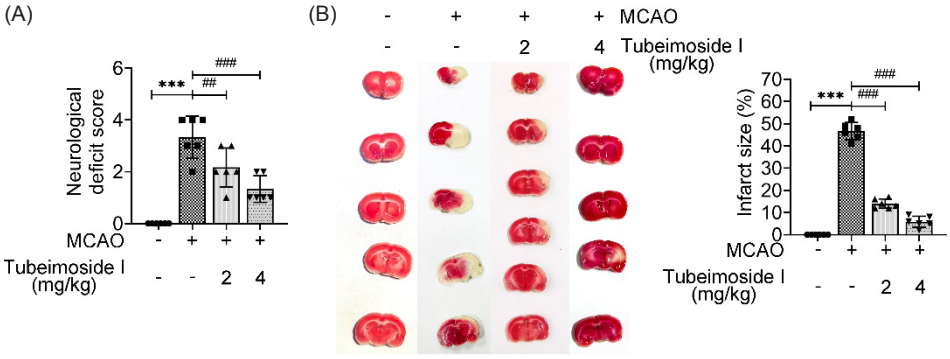

Figure 2. Tubeimoside I attenuates cerebral infarction in MCAO mice. (A) Neurological function score. (B) TTC staining and quantification of cerebral infarct area. ***P < 0.001 vs control group; ##P < 0.01 and #P < 0.05 vs MCAO group.

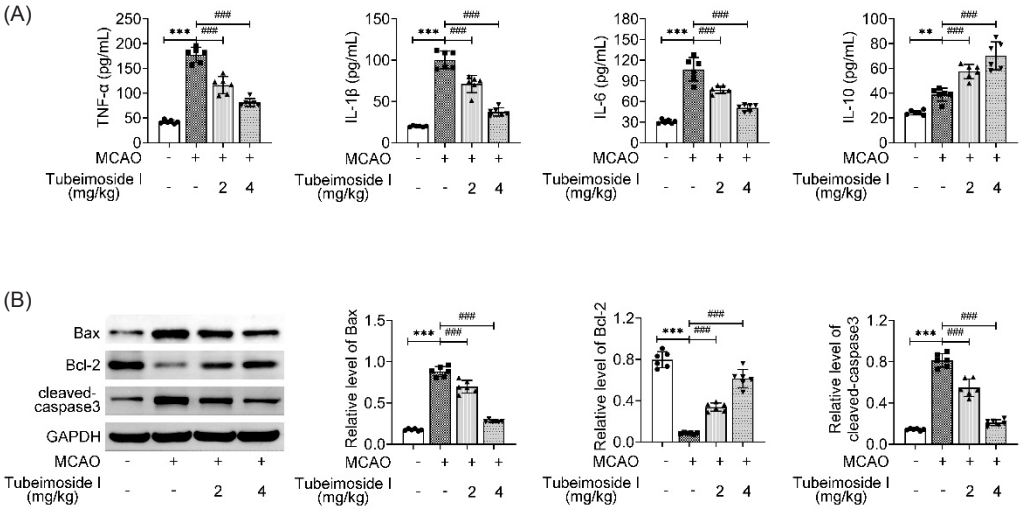

Figure 3. Tubeimoside I attenuates inflammation and apoptosis in MCAO mice. (A) Serum TNF-α, IL-1β, IL-6, and IL-10 levels in mice. (B) Bax, Bcl-2, and cleaved-caspase-3 protein expressions in mouse brain tissue. ***P < 0.001 and **P < 0.05 vs control group; ###P < 0.001 vs MCAO group.

Tubeimoside I activates SIRT3 *in vivo* and *in vitro*

We investigated SIRT3 expression in mouse brain tissues. We observed that the expression of SIRT3 was decreased in the brain tissues of MCAO mice whereas the same was increased in Tubeimoside I group, indicating that Tubeimoside I enhanced SIRT3 expression *in vivo* (Figure 4A). In addition, we examined SIRT3 expression in OGD/R-treated cells, and observed a decreased SIRT3 expression in OGD/R-treated cells whereas the same was increased in the cells of Tubeimoside I group, indicating that *in vitro* Tubeimoside I activated SIRT3 expression (Figure 4B).

Tubeimoside I attenuates OGD/R-induced cell injury by activating SIRT3

We added 3-(1H-1,2,3-triazol-4-yl) pyridine (3-TYP; a SIRT3 inhibitor) to Tubeimoside I-treated cells and examined cell viability and apoptosis. We discovered that 3-TYP reversed the ameliorative effects of Tubeimoside I on cell viability (Figure 5A) and apoptosis (Figures 5B and 5C), suggesting that Tubeimoside I ameliorated OGD/R-induced cell damage by activating SIRT3.

Discussion

In the current research, we determined the protective effects of Tubeimoside I against CIR injury *in vitro* and *in vivo*. Tubeimoside I increased OGD/R-induced cell viability and decreased apoptosis *in vitro*. On the other hand, Tubeimoside I increased neurological function scores, improved cerebral infarction, and decreased inflammation and apoptosis *in vivo*. In addition, ability of Tubeimoside I to prevent brain ischemia-reperfusion injury was linked to its activation of SIRT3 expression.

A major factor in CIR injury is inflammation. Studies have shown that inflammation accompanies the entire course of CIR injury, and neuroinflammation leads to neuronal damage (Petrovic-Djergovic *et al.*, 2016). TNF-α, IL-1β, and IL-6 are important inflammatory cytokines involved in the progression of CIR injury (Liu *et al.*, 2016); however, IL-10 is an anti-inflammatory factor that increases at the time of injury to counteract external stimuli.

The anti-inflammatory effect of Tubeimoside I has been reported previously, and pretreatment with Tubeimoside I significantly reduced inflammatory cell infiltration and prevented the generation of TNF-α, IL-6, and IL-1β in mice with acute lung injury (Wu *et al.*, 2013). In our study, serum TNF-α, IL-1β, IL-6, and IL-10 levels were significantly increased in MCAO group, while Tubeimoside I decreased serum TNF-α, IL-1β, and IL-6 levels but increased IL-10 levels; the results were consistent with that of previous studies.

The degeneration and apoptosis of neuronal cells caused by the release of pro-inflammatory cytokines is one of

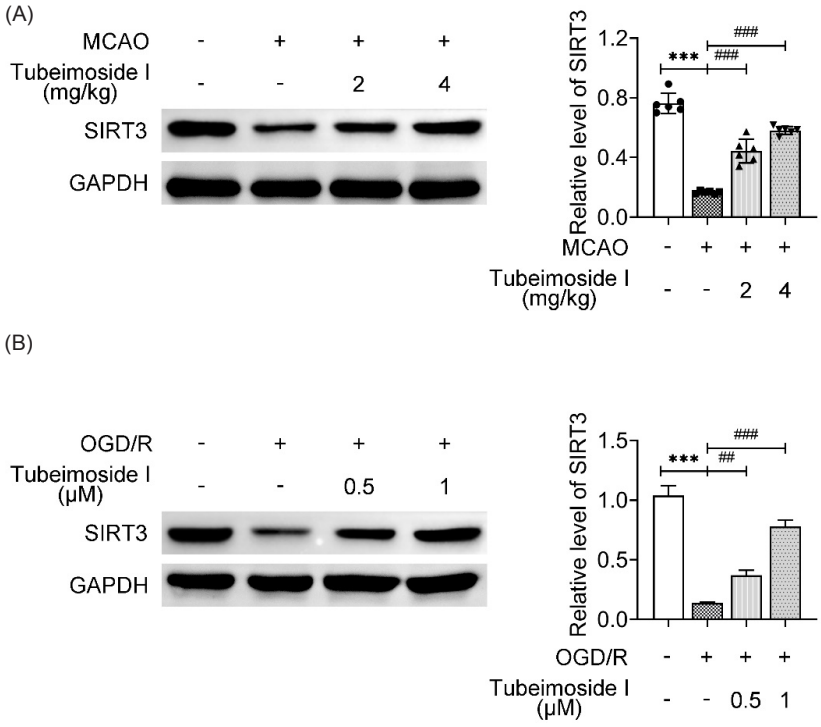

Figure 4. Tubeimoside I activates SIRT3 in vivo and in vitro. (A) Tubeimoside I enhances SIRT3 expression in vitro. (B) Tubeimoside I enhances SIRT3 expression *in vivo*. ***P < 0.001 vs control group; ###P < 0.001 and ##P < 0.01 vs MCAO group.

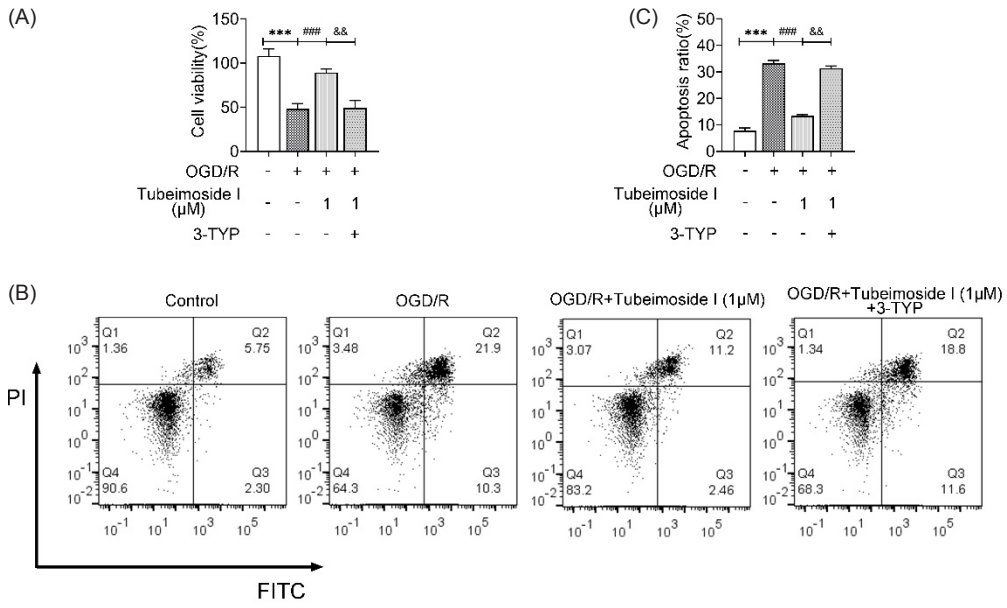

Figure 5. Tubeimoside I attenuates OGD/R-induced cell injury by activating SIRT3. (A) Addition of 3-YP reverses the promotive effect of Tubeimoside I on cell viability. (B) Addition of 3-YP reverses the inhibitory effect of Tubeimoside I on apoptosis. (C) Rate of apoptosis. ***P < 0.001 vs control group; ###P < 0.001 vs OGD/R group; &&P < 0.01 vs OGD/R + Tubeimoside I group.

the most significant causes of cell death in CIR damage (Pan *et al.*, 2021). It was established that cerebral ischemia caused Bax to relocate outside of mitochondrial membrane and increased outer mitochondrial membrane's permeability, which activated the intrinsic apoptotic pathway (Hu *et al.*, 2017). As the permeability of mitochondria's outer membrane increased, cytochrome C was released into cytoplasm, initiating the activation of Caspase-3 cascade reaction and ultimately causing DNA degradation and brain damage after ischemia. By maintaining the mitochondrial membrane potential and limiting the release of cytochrome C during this process, Bcl-2 suppressed apoptosis (Zhang *et al.*, 2019). In our study, the expression of Bax and cleaved-caspase-3 were increased and the expression of Bcl-2 was decreased in the brain tissue of MCAO mice, indicating that apoptosis occurred in brain tissue cells. However, Tubeimoside I decreased Bax and cleaved-caspase-3 expression and increased Bcl-2 protein expression, indicating that Tubeimoside I ameliorated apoptosis in brain tissue cells caused by CIR injury.

There are seven primary members of the class of nicotinamide adenine dinucleotide plus (NAD+)-dependent deacetylases known as sirtuins, which are primarily located in mitochondria (SIRT1 to SIRT7). Sirtuins are associated with a range of biological functions, including cell growth, metabolism, apoptosis, inflammation, and oxidative stress. SIRT3 has been reported to improve brain injury in CIR mice (Novgorodov *et al.*, 2016). SIRT3 expression is downregulated in CIR injury whereas its overexpression reduces brain infarct size and inhibits CIR injury-mediated neuronal apoptosis (Zhao *et al.*, 2018). Tubeimoside I activates SIRT3 to improve disease has been reported in several studies. Tubeimoside I improves endothelial function in sepsis by activating SIRT3 (Yang *et al.*, 2021a). In our study, Tubeimoside I enhanced SIRT3 expression both *in vivo* and *in vitro*, and rescue experiments revealed that SIRT3 inhibitor reversed the protective effect of Tubeimoside I on OGD/R-stimulated cells, suggesting that Tubeimoside I lessened CIR injury by activating SIRT3.

Conclusion

To sum up, our study demonstrated for the first time that Tubeimoside I activated SIRT3 to protect against CIR damage. This could be a new therapeutic strategy to counter CIR injury.

Availability of data and materials

All data generated and analyzed in this study are included in this published article. The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

Competing interests

The authors stated that there was no conflict of interest to disclose.

Ethics approval

Ethical approval was obtained from the Ethics Committee of Cangzhou Central Hospital.

All animal experiments were conducted in accordance with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines, the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines, and the EU Directive 2010/63/EU for animal experiments.

Author contributions

All authors contributed to the study's conception and design. Material preparation and experiments were performed by Shaoyue Huang. Data collection and analysis was performed by Zhen Hong. The first draft of the manuscript was prepared by Kuo Li. All authors checked and commented on different versions of the manuscript. All authors read and approved the final manuscript.

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