Shenmai Injection Exerts Neuroprotective Functions by Down-regulating MicroRNA-19a in H2O2-induced PC12 Cells

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Research Article

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Abstract

Background: Acute ischemic stroke (AIS) and following reperfusion therapy-induced cerebral ischemia reperfusion (I/R) injury have been recognized as an important subject of cerebrovascular disease with high mortality. Oxidative stress is an important pathological process of cerebral I/R injury. microRNA-19a (miR-19a) is involved in I/R. As the organ protectant agent, Shenmai Injection (SMI) is widely used in the clinical treatment of cerebral infarction.

Purpose: This study aims to explore whether SMI can reduce oxidative stress by regulating miR-19a, thereby treating I/R injury.

Methods: The oxidative stress state of PC12 cells was induced by \( \mathrm{H}_2\mathrm{O}_2 \), and then the cells were cultured with SMI. The therapeutic effect of SMI was evaluated by detecting cellular superoxide dismutase (SOD), malondialdehyde (MDA) and other oxidative markers with the kit. Western blot, PCR, immunofluorescence and other techniques were used to elucidate the potential mechanism of SMI.

Results: Cell viability assay results showed that SMI could improve the viability of PC12 cells stimulated by \( \mathrm{H}_2\mathrm{O}_2 \). Compared with the \( \mathrm{H}_2\mathrm{O}_2 \) group, after SMI treatment, the contents of MDA and reactive oxygen species (ROS) were significantly reduced, while the activity of SOD was significantly increased, and SMI could reduce apoptosis by increasing the content of adenosine 5’-triphosphate (ATP) in cells and enhancing the mitochondrial membrane potential (\( \Delta \Psi_m \)). Western blot and qRT-PCR results showed that these effects were partially achieved through the AMPK/Sirt1/PGC-1\( \alpha \) pathway. The level of miR-19a was significantly increased in \( \mathrm{H}_2\mathrm{O}_2 \) group, and SMI could protect the cells by reducing miR-19a. Further investigated the target of miR-19a, and transfected cells with miR-19a mimic and inhibitor respectively. We found that AdipoR2 was a direct target of miR-19a, and miR-19a could inhibit AdipoR2/PI3K/Akt/mTOR pathway.

Conclusion: SMI can activate AMPK/Sirt1/PGC-1\( \alpha \) and AdipoR2/PI3K/Akt/mTOR pathways by reducing miR-19a levels, and protect PC12 cells stimulated by \( \mathrm{H}_2\mathrm{O}_2 \).

1. Introduction

AIS is an important contributor to mortality and severely affects human health. Timely restoration of the blood supply is considered the main therapy for AIS. However, the reperfusion process following ischemic attack may further aggravate brain injury, known as cerebral I/R injury.

Although the exact mechanisms of I/R injury remain not fully clarified, it is increasingly recognized that a variety of pathophysiologic processes, including oxidative stress, inflammation, mitochondrial dysfunction, and apoptosis play a key role in the occurrence and development of I/R injury. Therefore, treatments based on the above mechanisms are considered as a promising strategy for alleviating the outcome of cerebral I/R injury.
SMI, as a traditional Chinese medicine injection, was approved by the China Food and Drug Administration (CFDA) in 1995 and has been widely used as an organ protectant in China. Consisting of aqueous extracts from two eminent Chinese Traditional medicine-Red ginseng (Hong Shen) and Ophiopogonis Radix (Mai Dong). Although Shenmai injection is widely used in the clinical treatment of cerebral infarction, but due to the lack of in-depth molecular biology studies and the complexity of its chemical components, the mechanism of action of SMI remains unclear.

microRNAs (miRs), as small non-coding RNA molecules, are known to regulate a variety of biological processes, including cell proliferation, neuroinflammation, and apoptosis. With the deepening of AIS studies, investigation of the role of miRs in I/R injury is increasing. miR-19a is a crucial member of the miR-17-92 cluster. Recent studies reported that miR-19a level increased after I/R, suggesting that miR-19a may be involved in I/R.

Taken together, this study aimed to explore the effect of SMI on miR-19a, its role in anti-oxidative stress and its possible related targets. To provide more evidence for the diagnostic markers of AIS and provide more methods for its treatment.

2. Materials And Methods

2.1 Chemicals and regents

SMI was provided by Zhengda Qingchunbao Pharmaceutical Co., Ltd. (Hangzhou, China). Fetal bovine serum and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco (Sacramento, USA). One Step TUNEL Apoptosis Assay Kit, Total Superoxide Dismutase (SOD) Assay Kit with WST-8, Lipid Peroxidation MDA Assay Kit, Reactive Oxygen Species (ROS) Assay Kit, ATP Assay Kit, and Mitochondrial membrane potential assay kit with JC-1 were purchased from Beyotime (Shanghai, China). Anti-AMPK alpha 1, Anti-SIRT1, Anti-PGC1 alpha, Anti-Bax, and Anti-Bcl-2 were from Abcam (Cambridge, MA, USA). Anti-AdipoR2 was purchased from Santa Cruz (California, USA). Anti-Pi3K, Anti-Akt, Antiphospho-Akt and Antiphospho-mTOR were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-MTOR, CoraLite488 - conjugated Affinipure Goat Anti-Mouse IgG(H+L), CoraLite488 – conjugated Affinipure Goat Anti-Rabbit IgG(H+L), and Anti-Beta Actin were purchased from Proteintech (Wuhan, China).

2.2 HPLC analysis of SMI

Since 2004, the CFDA has promulgated the "Drug Specifications", requiring all TCM injections to be standardized through chromatographic fingerprints before being marketed, and the national drug standard (WS3-B-3428-98-2010Z) issued by CFDA includes the revised standard of SMI. The composition of SMI was analyzed by high performance liquid chromatography (HPLC). Column: Water symmetry shieldTM RP18 column (4.6mm×250mm; 5.0 µm); column temperature: 30°C; mobile phase: acetonitrile (A)-water (B), gradient elution (0-30 min, 0% A→10% A; 30-40 min, 10% A→23% A; 40-50 min, 23% A; 50-
85 min, 23% A→60% A; 85-95 min, 60% A→100% A); flow rate: 1 mL/min; detection wavelength: 203 nm; injection volume: 10 µL.

2.3 Cell Cultures and Cell Treatment

The neuron-like rat pheochromocytoma cell line PC12 cells were obtained from Zhong Qiao Xin Zhou Biotechnology Co (Shanghai, China). The cells were cultured in DMEM medium containing 10% FBS in a humidified incubator (5% CO2, 37°C). The stimulating method of oxidative stress was to add H2O2 to the glucose-free medium, and cultured the cells for 24 h, then added SMI and continue to cultured the cells for 24h.

2.4 Cell transfection

The miR-19a mimic, inhibitor and their negative control were synthesized by GenePharma Co., Ltd. (Shanghai, China). Small interference RNA against AMPKα1 (si-AMPKα1) was purchased from Santa Cruz Biotechnology, Inc. (California, USA). Before H2O2, PC12 cells were seeded in 6-well plates and transfected with mimic, mimic-NC, inhibitor, inhibitor-NC, Si-AMPKα1, or empty vector respectively. All these transfection procedures were performed for 24h using Lipofectamine 2000 (ThermoFisher Scientific, Shanghai, China) following the manufacturer’s protocols. Then harvested the cells to perform follow-up experiments as indicated.

2.5 CCK-8 Assay.

To determine the appropriate concentration of H2O2 and SMI for further investigation in the subsequent experiments, cell viability was measured by the CCK-8 test. Briefly, 10µL CCK-8 was added into each well and incubated for 2 h. At last, the absorbance measurements were detected at 450 nm using an automatic microplate reader (Bio-Tek, USA).

2.6 TUNEL Staining

PC12 cells were seeded into 6-well culture plates. After treatment, cells were fixed with 4% paraformaldehyde for 30 minutes. Then use 0.3% Triton X-100 PBS incubated for 5 minutes. After incubated with TUNEL detection solution at 37°C for 1 hour. Images were captured using a fluorescence microscope (BX71, Olympus, Tokyo, Japan).

2.7 ROS Generation and Mitochondrial membrane potential assays
Briefly, the cells were seeded in 6-well plates, using the corresponding commercial kits according to the manufacturer’s protocols. Images were captured using a fluorescence microscope as previously described.

2.8 Determination of MDA, SOD, and ATP

The activities of MDA, SOD and ATP in cells were assessed using the corresponding commercial kits according to the manufacturer’s protocols, using a Microplate Reader to measure the value. Levels were standardized using total cellular protein determined by BCA assay.

2.9 Immunofluorescence staining

PC12 cells were fixed with 4% paraformaldehyde for 30 min and then permeabilized with 0.3% Triton X-100. After blocking with 5% BSA Blocking Buffer, the cells were incubated with AMPK alpha 1 antibody or AdipoR2 antibody overnight at 4°C. Next, cells were incubated with CoraLite 488-conjugated secondary goat anti-mouse IgG antibody at 37°C for 1 h and DAPI for 5 min. Fluorescence images were acquired by a fluorescence microscope.

2.10 Western Blotting Analysis

After corresponding treatments, protein extracts were isolated from each group of cells using RIPA protein lysis buffer containing protein phosphatase inhibitors. The concentration of total protein was measured via the BCA Protein Quantification Kit according to the manufacturer’s protocol. Equal amounts of protein samples (30 µg) were separated by SDS-PAGE and transferred to the polyvinylidene difluoride (PVDF) membranes. The corresponding membranes were blocked for 1 h and then incubated with primary antibodies and subsequently incubated with secondary antibodies for 1 h at room temperature. The ECL developer was added, then the protein-antibody complexes were photographed and analyzed by Image J software.

2.11 qRT-PCR Experiment

Total cellular RNA was isolated by TRIzol reagent (Invitrogen, USA). Used the Applied Biosystems 7300 Fast Real-Time PCR System (Thermo Fisher Scientific, USA), according to the manufacturer’s protocols, in the 20ul reaction volume, first reversely transcribed RNA into complementary DNA (cDNA), and then carried out the quantitative real-time PCR (qRT-PCR). All primers were designed and synthesized by Shanghai Sangon Biotech in China. miR-19a expression was determined using Hairpin-itTM miRNAs RT-PCR Quantitation Kit (GenePharma, China) with U6 as an internal reference. The specific sequences of every pair of primers were available in Table S1. The relative amount of gene expression was calculated by $2^{-\Delta\Delta CT}$. All experiments were repeated three times to intensify the credibility.
2.12 Statistical Analysis

The data were presented as mean±SD. Data were analyzed using GraphPad Prism software (version 8.0.1). The $P$ value was calculated using one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

3. Results

3.1 Chemical characterization of SMI

The quality control of the fingerprint of Shenmai Injection requires that 16 characteristic peaks corresponding to the standard fingerprint of Shenmai injection should be presented within 8-90 min. And clarify the three characteristic peak components, namely Ginsenoside Rb1, Ginsenoside Rg1 and Ginsenoside Re (figure 1).

3.2 SMI Protects the Cell Viability of H$_2$O$_2$-Stimulated PC12 Cells

The CCK-8 method was used to detect the effects of different concentrations of H$_2$O$_2$ and SMI on the cells. As shown in figure 2(a,b), the higher concentration of H$_2$O$_2$, the lower cell survival rate, while the SMI of 0-100ul/ml has no significant effect on the cell survival rate. When the concentration of H$_2$O$_2$ is 100uM, the cell survival rate is 45.56%. Therefore, in the following experiments, 100uM H$_2$O$_2$ is used to stimulate the cells as a pathological model of oxidative stress. After stimulation with 100uM H$_2$O$_2$, treatment was performed with different concentrations of SMI. After 20ul/ml SMI was applied, the cell survival rate was the highest (figure 2(c)), so 20ul/ml SMI was used for subsequent experiments.

3.3.1 SMI Ameliorated cells Injury by Enhancing AMPKα1 Level

To clarify the mechanism of SMI, the expression of AMPKα1 was measured by immunofluorescence method, and to further investigate whether AMPKα1 plays the key role in SMI treatment, we used AMPKα1-specific siRNA to significantly decrease AMPKα1. As shown in figure 3, SMI significantly enhanced the decreased fluorescence intensity due to H$_2$O$_2$ stimulation, and this effect was reversed by Si-AMPKα1. SiRNA-NC had no obvious effect on cells.

3.3.2 Effect of SMI on the AMPKα1 /Sirt1/PGC-1α Signal Pathway in PC12 Cells Stimulated by H$_2$O$_2$
The AMPKα1/Sirt1/PGC-1α signaling pathway plays an important role in metabolizing energy\[^{[1]}\]. qRT-PCR (figure 4(a,b,c)) and western blot (figure 4(d,e,f,g,)) showed the same trend of results, H\(_2\)O\(_2\) significantly decreased the expression of AMPKα1, SIRT1, and PGC-1α, compared with the control group. SMI treatment could significantly upregulate the expression of AMPKα1, SIRT1, and PGC-1α in PC12 cells. However, the trend of SMI increasing AMPKα1, SIRT1, and PGC-1α was reversed to varying degrees after the cells were transfected with Si-AMPKα1. These results showed that SMI may have a protective effect on H\(_2\)O\(_2\)-induced PC12 cells via the AMPKα1/Sirt1/PGC-1α pathway.

### 3.4.1 SMI Attenuated H\(_2\)O\(_2\)-Induced PC12 Cells Apoptosis through AMPKα1

TUNEL assay was performed to detected cell apoptosis. Compared with the control group, the proportion of TUNEL-positive cells (red fluorescence) treated with H\(_2\)O\(_2\) were significantly increased (figure 5(a)). Treatment with SMI significantly reduced the apoptotic index compared to the H\(_2\)O\(_2\) group.

Bax and Bcl2, as proapoptotic protein and antiapoptotic protein, played crucial roles in regulating cell apoptosis. In the H\(_2\)O\(_2\) group, the protein expression of Bcl2/Bax was markedly lower compared to the control group, and were ameliorated by treatment with SMI (figure 5(b,c)). These results were consistent with the TUNEL assay.

Furthermore, the effects of SMI on PC12 cell apoptosis were blocked by Si-AMPKα1. These data further supported the hypothesis that the AMPKα1 played an essential role in the antiapoptosis mechanism of SMI.

### 3.4.2 SMI Attenuated H\(_2\)O\(_2\)-Induced PC12 Cells Oxidative Stress through AMPKα1

To explore the possible mechanism of SMI on H\(_2\)O\(_2\)-induced injury in PC12 cells, we examined the biochemical markers of oxidative stress, including MDA, ROS, and SOD activities. H\(_2\)O\(_2\) could dramatically increase the MDA, ROS levels and decrease the activity of SOD, compared with the control group (figure 6). However, SMI treatment could significantly increase the SOD activity and decrease the levels of MDA, ROS. In contrast, the improvement of SOD activity and MDA, ROS level was suppressed by Si-AMPKα1. All the above results demonstrated that SMI could markedly alleviate H\(_2\)O\(_2\)-induced oxidative abnormalities in PC12 cells, whereas the antioxidative activity was attenuated by Si-AMPKα1.

### 3.4.3 SMI Attenuated H\(_2\)O\(_2\)-Induced PC12 Cells Mitochondrial damage through AMPKα1
Assess cell mitochondrial function by detecting adenosine 5'-triphosphate (ATP) and mitochondrial membrane potential (ΔΨm) levels. SMI treatment could significantly change the JC-1 fluorescent probe from green fluorescence to red fluorescence in PC12 cells, and increase the ATP levels (figure 7), compared with the H₂O₂ group. But the effect of SMI was partially reversed by Si-AMPKα1. The results showed that SMI could significantly alleviate the mitochondrial dysfunction of PC12 cells via increasing ATP and ΔΨm, and the mechanism of action is related to AMPKα1.

3.5 SMI declined the miR-19a expression in H₂O₂-Induced PC12 cells

Considering that miR-19a was identified as a sign of nerve injury, we investigated whether miR-19a was associated with H₂O₂ induced cells. qRT-qPCR analysis showed that the H₂O₂ exposed PC12 cells significantly induced miR-19a expression compared with the control group (figure 8(a)). By contrast, SMI overturned the acceleration of H₂O₂ on miR-19a expression. These results manifested that miR-19a might either be involved in or serve as an effector of H₂O₂ injury, and SMI could restrain miR-19a expression in H₂O₂-stimulated PC12 cells.

3.6.1 SMI Regulated the Effects of AMPKα1 in H₂O₂-Induced PC12 cells Through miR-19a

The above experimental results showed that the therapeutic effect of SMI was related to both AMPKα1 and miR-19a. Therefore, to verify whether miR-19a had a regulatory effect on AMPKα1, we transfected cells with miR-19a mimic and inhibitor respectively, and observed the effect of increased and silenced miR-19a expression on AMPKα1 by immunofluorescence method.

As shown in Figure 8(b), cells transfected with miR-19a mimic and then subjected to H₂O₂ stimulation and SMI treatment showed significantly reduced AMPKα1 fluorescence intensity compared with the cells that were directly subjected to H₂O₂ and SMI without transfection, indicating that high expression of miR-19a could reduce the expression of AMPKα1. On the other hand, the fluorescence intensity of AMPKα1 in cells transfected with inhibitor and then stimulated with H₂O₂ was not significantly different from that of cells directly treated with H₂O₂ and SMI without transfection, indicating that SMI and inhibitor had similar effects, that is, SMI it can play a therapeutic role by reducing the expression of miR-19a.

3.6.2 SMI activated AMPKα1/Sirt1/PGC-1α signaling pathway via reducing miR-19a expression
To further clarify the relationship between miR-19a and AMPKα/Sirt1/PGC-1α pathway, cells were transfected with miR-19a mimic and inhibitor, respectively, to observe the effect of increased and decreased expression of miR-19a on AMPKα/Sirt1/PGC-1α pathway. The results showed that the increased expression of miR-19a could offset the activation of AMPKα/Sirt1/PGC-1α pathway by SMI (figure 9), while the decreased expression of miR-19a had similar activation of AMPKα/Sirt1/PGC-1α pathway by SMI. The above results indicated that SMI improved cell oxidative stress injury through miR-19a/AMPKα/Sirt1/PGC-1α pathway.

3.7.1 Down-Regulation of miR-19a Caused by SMI Inhibited Cell Apoptosis

As mentioned above, the apoptosis level was assessed by tunnel assay and the expression of Bcl2/Bax protein. Compared with the cells directly treated with H2O2 and SMI, the cells transfected miR-19a with mimic showed higher fluorescence intensity and lower ratio of Bcl2/Bax (figure 10), indicating that mimic reversed the protective effect of SMI. However, cells transfected with inhibitor and then stimulated with H2O2, the apoptosis rate was lower than that of untransfected cells, indicating that inhibitor could reduce the apoptosis induced by H2O2, but its protective effect was lower than that of SMI.

3.7.2 Down-Regulation of miR-19a Caused by SMI Inhibited Cell Oxidative Stress

Compared with untransfected cells, the MDA content and ROS fluorescence intensity of mimic-transfected cells were significantly increased after H2O2 and SMI (figure 11(a,c)), and the SOD content was significantly decreased (figure 11(b)), indicating that the oxidative stress of cells was enhanced. Inhibitor and SMI have similar effects on reducing cellular oxidative stress, but SMI was more effective.

3.7.3 Down-Regulation of miR-19a Caused by SMI Inhibited Cell Mitochondrial damage

Compared with cells transfected with mimic, more JC-1 fluorescent probes changed from red light to green light in ΔΨm detection, and the ATP content was also reduced (figure 12). The cells were transfected with inhibitor and then stimulated with H2O2, compared with the cells directly stimulated with H2O2 without transfection, the JC-1 fluorescent probe changed more from green to red, and the ATP content also increased. The results showed that miR-19a had a protective effect on Mitochondria function.
3.8.1 SMI Regulated the Effects of AdipoR2 in H$_2$O$_2$-Induced PC12 cells Through miR-19a

To further investigate the mechanisms miR-19a silencing modulated apoptosis, we predicted the putative gene targets of miR-19a silencing by using the TargetScan software. Among all of the predicted gene targets, encoding for adiponectin receptor 2 (AdipoR2) was chosen as a candidate for two reasons. First, in the brain, adiponectin signaling directly affects important brain functions, such as energy balance, synaptic plasticity, and hippocampal neurogenesis, and through its receptors AdipoR1 and AdipoR2\(^\text{[1]}\). Secondly, despite the association between serum adiponectin levels and stroke risk or stroke rehabilitation is unclear, several studies have suggested that adiponectin mediated mechanisms have a protective effect on atherosclerosis and stroke pathogenesis\(^\text{[2]}\). Hence, we used immunofluorescence to confirm whether AdipoR2 is a direct target of miR-19a.

As shown in Figure 13, SMI can significantly increase the fluorescence intensity of AdipoR2 weakened by H$_2$O$_2$, while the effect of SMI on AdipoR2 was reversed by miR-19a mimic. On the other hand, miR-19a inhibitor can increase the fluorescence intensity of AdipoR2 just like SMI. The above results indicate that AdipoR2 is one of the direct targets of 19a, and SMI can increase the expression of AdipoR2 by reducing the expression of miR-19a.

3.8.2 SMI activated AdipoR2/PI3K/Akt/mTOR signaling pathway via reducing miR-19a expression

After confirmed that SMI can regulate AdipoR2 through miR-19a, the western blot method was used to further determine whether SMI can regulate AdipoR2/PI3K/Akt/mTOR pathway through miR-19a (figure 14). Compared with H$_2$O$_2$ group, SMI significantly increased the expression of genes in the AdipoR2/PI3K/Akt/mTOR pathway, and this effect was significantly reversed by miR-19a mimic. After H$_2$O$_2$ stimulation, SMI and miR-19a inhibitor had similar effects, both activating the AdipoR2/PI3K/Akt/mTOR pathway, but SMI seemed to have a better effect, suggesting that AdipoR2/PI3K/Akt/mTOR may be one of the pathways through which SMI passes through miR-19a.

4. Discussion

SMI is a Chinese patent medicine injection widely used in the clinic. In previous studies, SMI and its main constituents, ginsenosides Rb1 and Rg1, reduced the release of lactate dehydrogenase (LDH) and improved the survival rate of cultured neurons, vascular endothelial cells, and astrocytes under hypoxia/hypoglycemia/reoxygenation injury\(^\text{[3]}\); helping maintain the integrity of the blood-brain barrier
(BBB) function during focal cerebral ischemic injury\[^{13}\]; protecting mitochondria from oxidative stress by increasing the level of PDH, suggesting that it may improve the energy metabolism of cardiomyocytes\[^{1}\].

In this study, SMI treatment obviously improved cell viability. Further experiments confirmed that SMI exerted an antioxidation effect by regulating MDA, SOD, and ROS levels or activities, affecting mitochondrial membrane potential and ATP contents, to prevented cell apoptosis as evidenced by increased Bcl2/Bax ratio, and decreased TUNEL-positive rate.

The AMP-activated protein kinase (AMPK), known as an "energy sensor" or "gauge," is expressed in all types of cells. Previous studies have reported that AMPK has a protective effect on global cerebral ischemia\[^{79}\]. Mammalian AMPK is a heterotrimeric complex composed of α, β, and γ subunits, which have enzymatic, scaffolding, and regulatory functions, respectively\[^{88}\]. As an important subtype of AMPK, AMPKα1 plays an important role in arteriogenesis and collateral remodeling, and its activation contributes to the recovery of occlusive vascular disease\[^{326}\]. AMPK stimulates the activity Sirt1 by providing substrate NAD\(^+\) to regulate the expression of downstream energy metabolism genes, including PGC-1α deacetylation\[^{104}\]. Interestingly, PGC-1α is not only a regulator of mitochondrial biosynthesis, but also an important administrator of oxidative metabolism\[^{285}\]. PGC-1α promotes the activity of antioxidant enzymes, such as SOD, and protects neurons from neutral death induced by oxidative stress\[^{465}\]. We have observed that SMI can stimulate the AMPKα1/Sirt1/PGC-1α signaling pathway in cell apoptosis induced by oxidative stress. Furthermore, the protective effect of SMI on PC12 cells was partially inhibited after transfection with Si-AMPKα1. Our results showed that SMI partially inhibited H\(_2\)O\(_2\)-induced oxidative stress and apoptosis via the AMPKα1/SIRT1/PGC-1 α signaling pathway.

miR-19a is a non-coding RNA located on human chromosome 14, with a length of about 82bp, which is directly related to the occurrence and development of a variety of diseases\[^{420}\]. Bioinformatics analysis revealed that miR-19a might be used as a diagnostic and prognostic biomarker for AIS\[^{486}\]. It has been reported that increased miR-19a promotes cerebral ischemic injury by regulating glucose uptake and neuronal apoptosis\[^{130}\].

Compared with previous studies, this study evaluated the effect of H\(_2\)O\(_2\) stimulation on miR-19a in PC12 cells. The results showed that SMI obviously reduced miR-19a expression, thereby attenuating PC12 damage in H\(_2\)O\(_2\) injured. Overexpression of miR-19a can reduce the protective effect of SMI on PC12, while silencing miR-19a can also have the same protective effect on cells as SMI. Collectively, these data indicated that SMI is an effective treatment that ameliorated oxidative stress damage by downregulating miR-19a in H\(_2\)O\(_2\)-induced apoptosis of PC12 cells, and this protective effect is associated with the AMPKα1/Sirt1/PGC-1 α pathway.

Based on the obtained results that SMI protected PC12 against apoptosis by regulating miR-19a, further experiments to identify the potential target of SMI were conducted. As adiponectin is associated with many cerebrovascular risk factors, such as type II diabetes, hypertension and changes in lipid
metabolism, a link between adiponectin and stroke is expected\[1\]. AdipoR2 inhibits the induction of intracellular cell adhesion molecule-1 and vascular cell adhesion molecule-1\[22\], which typically bind to leukocytes and initiate atherosclerosis following endothelial cell injury\[\]\. PI3K/Akt/mTOR signaling pathway is considered to be a crucial regulator of apoptosis cell survival, proliferation, growth, angiogenesis, translation, transcription and metabolism\[\]\. In the AdipoR2 signaling pathway, AdipoR2 activates PI3K, which triggers AKT, the activation of AKT inhibits apoptosis\[\], mTOR regulates steroid biosynthesis, mediates the responses to hypoxia by enhancing the transcription of hypoxia-inducible factor 1α (HIF1α), and is involved in epithelial-mesenchymal transition and new angiogenesis\[\]. Our study showed for the first time the changes in the expression of Adipor2 in the \(\text{H}_2\text{O}_2\) injury model, and demonstrated that SMI can regulate the AdipoR2/PI3K/Akt/mTOR pathway via reducing the expression of miR-19a.

5. Conclusions

Taken together, we have confirmed that SMI protected PC12 against \(\text{H}_2\text{O}_2\) injury and suppressed oxidative stress, mitochondrial dysfunction and apoptosis. More importantly, we found that downregulation of miR-19a inhibits PC12 apoptosis and might attenuate \(\text{H}_2\text{O}_2\) injury. All these results indicated that the protective effect of SMI on PC12 is at least partially mediated by down-regulation of miR-19a, which activates AMPKα1/Sirt1/PGC-1α and AdipoR2/PI3K/Akt/mTOR pathways and thus inhibits apoptosis. These findings demonstrated that miR-19a, AMPKα1/Sirt1/PGC-1α pathway, and AdipoR2/PI3K/Akt/mTOR pathway might be potential therapeutic targets for \(\text{H}_2\text{O}_2\) injury treatment, and it might also lay the foundation for the further development of new drugs for AIS.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent to publish

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests
The authors declare no conflict of interest.

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**Authors' Contributions**

Jing Wu: Data curation, perform the experiments, Writing review & editing. Zhonghao Li: Data curation, Investigation, Methodology. Xiaoke Dong: Data curation, Methodology. Siyuan Yuan: Data curation, Methodology. Le Wang: Data curation, Methodology, Funding acquisition. Jinmin Liu: Supervision, Formal analysis.

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Figures
Figure 1

HPLC analysis of SM injection

Figure 2

SMI treatment enhances the viability of cells induced by H$_2$O$_2$. (a,b) Effects of different concentrations of H$_2$O$_2$ and SMI on the viability of PC12 cells. (c) Effects of treatment with different concentrations of SMI on the viability of PC12 cells induced by H$_2$O$_2$. **$P<0.01$ compared with control group; *$P<0.05$ compared with control group; ##$P<0.01$ compared with H$_2$O$_2$ group. “+”: with the treatments in cells; “−”: without the treatments in cells.
Figure 3

Immunofluorescence images showed the effect of SMI on AMPKα1 expression. Scale bar = 20 μM.
Figure 4

SMI alleviated H$_2$O$_2$ injury in PC12 cells through AMPKα1/Sirt1/
P65 Signal Pathway. (a,b,c) RNA levels detected by qRT-PCR; (d,e,f,g) Protein levels detected by western blot. **$P<0.01$ compared with control group; *$P<0.05$ compared with control group; #P < 0.05; ##P < 0.01.
SMI Attenuated H$_2$O$_2$-Induced PC12 Cells Apoptosis through AMPKα1. (a) Apoptosis was detected by TUNEL, Scale bar =20μM; (b,c) Western blot detection of Bcl2/Bax protein expression. **$P<0.01$ compared with control group; *$P<0.05$ compared with control group; ##$P<0.01$. 

Figure 5
Figure 6

SMI Attenuated H₂O₂-Induced PC12 Cells Oxidative Stress through AMPKα1. (a,b) The expression levels of MDA and SOD in PC12 cells; (c) Fluorescent image of ROS in PC12 cells, Scale bar =20μM. **P <0.01 compared with control group; *P <0.05 compared with control group; #P < 0.05; ##P < 0.01.

Figure 7

SMI Attenuated H₂O₂-Induced PC12 Cells Mitochondrial damage through AMPKα1. (a) Fluorescence image of mitochondrial membrane potential detected by ΔΨm, Scale bar =20μM; (b) The expression levels of ATP in PC12 cells. **P <0.01 compared with control group; *P <0.05 compared with control group; #P < 0.05; ##P < 0.01.
Figure 8

Effect of miR-19a content on PC12 cells. (a) effect of SMI on miR-19a levels; (b) Immunofluorescence image of miR-19a effect on AMPKα1. Scale bar =20μM
Figure 9

Western blot detection of AMPKα1/Sirt1/PGC-1α pathway protein expression. **$P<0.01$ compared with control group; *$P<0.05$ compared with control group; # $P < 0.05$; ## $P < 0.01$. 
Figure 10

SMI Inhibits apoptosis by down-regulation miR-19a. (a) Apoptosis was detected by TUNEL, Scale bar =20μM; (b,c) Western blot detection of Bcl2/Bax protein expression. **P < 0.01 compared with control group; *P < 0.05 compared with control group; #P < 0.05; ##P < 0.01.
Figure 11

SMI inhibits Oxidative Stress by down-regulating miR-19a. (a,b) The expression levels of MDA and SOD in PC12 cells; (c) Fluorescent image of ROS in PC12 cells, Scale bar = 20 μM. **P < 0.01 compared with control group; *P < 0.05 compared with control group; #P < 0.05; ##P < 0.01.
Figure 12

SMI Attenuated H$_2$O$_2$-Induced PC12 Cells Mitochondrial damage through miR-19a. (a) Fluorescence image of mitochondrial membrane potential detected by ΔΨm, Scale bar =20μM; (b) The expression levels of ATP in PC12 cells. **$P<0.01$ compared with control group; *$P<0.05$ compared with control group; #P < 0.05; ##P < 0.01.
Figure 13

Immunofluorescence image of AdipoR2, Scale bar = 20 μM.
Figure 14

Western blot detection of AdipoR2/PI3K/Akt/mTOR pathway protein expression. **P < 0.01 compared with control group; *P < 0.05 compared with control group; #P < 0.05; ##P < 0.01.
Figure 14

Western blot detection of AdipoR2/PI3K/Akt/mTOR pathway protein expression. **P < 0.01 compared with control group; *P < 0.05 compared with control group; #P < 0.05; ##P < 0.01.

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