Typhae pollen polysaccharides protect hypoxia-induced PC12 cell injury via regulation of miR-34a/SIRT1

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Abstract
This current research was performed to investigate the role of typhae pollen polysaccharides (TPP) in hypoxia-treated PC12 cell which was an in vitro cell model of cerebral ischemia. Hypoxia-treated cells were treated with TPP for 12 h. Cell viability and apoptosis were detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay and flow cytometry, respectively. Cell apoptotic proteins and PI3K/AKT and Ras/Raf/MEK/ERK signal pathway–associated proteins were also examined by western blot. Furthermore, abnormal expression of miR-34a and silent information regulator 1 (SIRT1) was achieved by transfection. Besides, the expression of miR-34a and SIRT1 was examined by quantitative real-time polymerase chain reaction (qRT-PCR). The expression of SIRT1 was detected by qRT-PCR and western blot. The relationship between miR-34a and SIRT1 was verified by luciferase assay. We found that TPP enhanced cell viability and inhibited apoptosis in hypoxia-treated PC12 cells. Moreover, TPP increased the accumulated levels of Bcl-2 while decreased expression of Bax, cleaved Caspase-3, and cleaved PARP. TPP downregulated miR-34a expression while induced by hypoxia. Further results showed that miR-34a overexpression reversed the results led by TPP in cell viability, apoptosis, and its related proteins. In addition, SIRT1 was upregulated by TPP and was verified to be a target of miR-34a. Silence of SIRT1 led to the opposite results led by TPP. In the end, TPP activated PI3K/AKT and Ras/Raf/MEK/ERK signal pathways. In conclusion, TPP plays important roles in regulating cell viability and apoptosis in hypoxia-treated PC12 cells via modulating miR-34a/SIRT1, as well as activating PI3K/AKT and Ras/Raf/MEK/ERK signal pathways.

Keywords
cerebral ischemia, miR-34a, PI3K/AKT, Ras/Raf/MEK/ERK, SIRT1, TPP

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Introduction
Cerebral ischemia, as an emergency, refers to the brain function disorder caused by the insufficient blood supply to a certain part of the brain.¹ This process leads to hypoxia and even death of brain tissue, which might cause cerebral infarction/ischemic stroke.² Ischemic stroke, as a complicated pathophysiology, normally brings serious results including irreversible neuronal damage and further involved in a series of biological processes for neuronal repair.³ Nowadays, even with the development of science and technology, the treatment of cerebral ischemia, especially for the acute type, the therapeutic options are still very limited. Therefore, exploring new medicine and novel treatment therapies is urgently required nowadays.
During the investigation for the treatment of cerebral ischemia, traditional Chinese medicine shed a light on the cerebral ischemia which has high morbidity and mortality.\(^4,5\) Typhae pollen (TP), first recorded in the oldest Chinese pharmacopoeia called “Shennong Bencao Jing”, is extracted from *Typha angustifolia* L., *Typha orientalis* Presl.\(^6\) It is a traditional Chinese medicine with a long history of use. It is used to treat clinical ophthalmic diseases such as fundus hemorrhage, blood circulation, and removing blood stasis.\(^5,7\) Importantly, TP has been proved to play important roles in treatment of hemorrhagic diseases\(^8\) and typhae pollen polysaccharides (TPP) were reported to improve hemorheology indexes,\(^6\) which motivated us that it might also function in the treatment of cerebral ischemia.

MicroRNAs (miRNAs) are involved in most of the biological disorders including cerebral ischemia, in which they function as potential diagnostic and therapeutic targets.\(^9\) For example, miR-124 was reported to have neuroprotective function and induced functional improvement after focal cerebral ischemia.\(^10\) It was revealed miR-128-3p exerted protective function in cerebral ischemia of mouse via inactivating p38\(\alpha\) mitogen-activated protein kinase.\(^11\) Interestingly, miR-34a is reported to participate in series of ischemia such as intestinal ischemia\(^12\) and hepatic ischemia.\(^13\) Moreover, one of the most significant current literatures showed that miR-34a demonstrated its crucial functions in brain. For example, miR-34a modulates blood–brain barrier and regulates mitochondrial functions via targeting cytochrome c.\(^14\) Then, the involvement of miR-34a in the progression of cerebral ischemia was also investigated.

Silent information regulator 1 (SIRT1), kind of NAD\(^+\)-dependent deacetylase, is a member of sirtuin family.\(^15\) By being regulated by C-terminal regulatory segment, SIRT1 is involved in the development and progression of several diseases by deacetylating transcriptional factors.\(^16\) Studies revealed that SIRT1 exerted indispensable suppressor functions by inhibiting cell behaviors, such as proliferation, invasion, oxidative stress, and inflammation, in fatty liver diseases, oral squamous cell carcinoma, and atherosclerosis.\(^17–19\) Accumulating evidences further demonstrated that SIRT1 relieved hypoxia or lipopolysaccharide-caused cell injury in cerebral ischemic models by impeding apoptosis and inflammatory response.\(^20,21\) Nevertheless, it still remains elusive whether SIRT1 participates in the regulation of TPP in cerebral ischemia.

The neurological damage, caused by cerebral ischemia, is a sophisticated pathophysiological process.\(^22\) In the past, neuronal cells were used to study cell structural and molecular functions.\(^23\) However, this was a challenging procedure due to the technical barriers to primary cell culture. PC12 cells, a kind of clonal line extracted from neural crest of rat pheochromocytoma,\(^24\) consist of neuroblast and oxyphil cells. It is precisely because of the neuronal origin and the ability to acquire functional characteristics; PC12 cells are diffusely applied in the researches about ischemic stroke to expand our knowledge on pathological process.\(^25,26\) In our study, PC12 cells were stimulated by hypoxia to establish an in vitro cell model of cerebral ischemia, and then the roles of TPP and the underlying mechanism were explored.

**Materials and methods**

**TPP preparation**

*Typha orientalis* was provided by Tongrentang Group Co., Ltd. (Xi’an, China). The polysaccharide was extracted following the subsequent steps. First, ethanol was used to remove extra impurity. After that, water extraction and alcohol precipitation approach were supplied to collect the polysaccharides. Then, Sevage method was used to remove free proteins from the polysaccharide solution.\(^6\) Finally, *T. orientalis* polysaccharides were obtained using vacuum freeze-drying method.

**Cell culture and treatment**

Kunming Institute of Zoology (Kunming, China) provided PC12 cells which were used in this study. The cells were cultured in DMEM (Dulbecco’s modified Eagle medium) with 10% (v/v) fetal bovine serum (FBS; Life Science, UT, USA), 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin and maintained in an environment with 37°C and 5% CO\(_2\). Then, the cells were placed in an incubator subchamber (Biospherix; New York, NY, USA) which was exposed with 3% O\(_2\) by Compact O\(_2\) and CO\(_2\) Subchamber Controller (Biospherix) for 12h. In addition, cells cultured in normoxic condition of 21% O\(_2\) were regarded as control group.
Moreover, TPP was diluted into concentration of 0–300 μM and cells were treated in TPP for 12 h.

**Cell viability assay**

First, cells were seeded in a 96-well plate and transfection was performed when cells achieved 50% confluence. Then, cells were cultured for 48 h; 20 μL of 5-mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was then added and maintained for 4 h. After that culture medium was removed and dimethyl sulfoxide (DMSO) was added in each well and mixed well. The optical density (OD) value was read at 560 nm to detect cell viability.

**Apoptosis assay**

The cells were seeded in a six-well plate. Cells were cultured until 50% confluence was achieved, and then cell transfection was performed. Following the manufacturer’s instruction of apoptosis detection kit, the cells were digested using trypsin and washed with phosphate-buffered saline (PBS), and then centrifuged for 5 min at the speed of 2000 r/min. Cells were collected and 500 μL binding buffer, 5 μL Annexin V-FITC, and 5 μL PI were added, mixed well, and maintained in darkness for 10 min. Flow cytometry by fluorescence-activated cell sorting (FACS) (Beckman Coulter, Fullerton, CA, USA) was used to detect cell apoptosis 1 h later.

**Western blot**

Radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) was added to the collected cells and shaken in vortex finder for 30 s. After 40 min, the liquid supernatant was collected after centrifuged for 12 min at 13,500 r/min at 4°C. The BCA (bicinchoninic acid) approach was used to determine the concentration of protein. About 10%–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was used to isolate the same amount of protein and transfer it to nitrocellulose membrane. First, antibodies were added after 5% skimmed milk and sealed for 1–1.5 h in room temperature. Then, they were washed with PBST (phosphate-buffered saline with Tween® 20) three times; secondary antibodies were used and incubated for 1 h. The Image Lab™ Software (Bio-Rad, Shanghai, China) was used to detect the western blot band and Odyssey 3.0 software to quantify the intensity of the bands.

**Quantitative real-time polymerase chain reaction**

According to trizol kit instruction, RNA was extracted. The whole extraction process was set under RNAase condition. Using qRT-PCR kit, RNA was made into complementary DNA (cDNA), and amplification was done. The 5 μL amplification products was obtained and used for SDS-PAGE western blot. Primer was added to 25 μL reaction systems and the condition was set as follows: 94°C for 45 s, 59°C for 45 s, and 72°C for 60 s.

**Transfection**

MiR-34a mimic and the NC mimic were synthesized (Life Technologies Corporation, Frederick, MD, USA) and transfected into cells in this study. For knockdown of SIRT1, si-NC and si-SIRT1 were transfected into cells using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). Cells were harvested after 48 h for the following experiments.

**Dual luciferase activity assay**

MiR-34a and SIRT1 were co-transfected into cells. Cells were washed with PBS for three times and then passive lysis buffer (PLB) lysate was added to lysate cells and then Luciferase Assay Reagent (LAR, Promega, Madison, WI, USA) was added. Dual luciferase activity assay was performed to read the fluorescence value before stop buffer was added. The fluorescence value was read again. Relative luciferase activity was counted in the end.

**Statistical analysis**

Data management and analysis were performed using Graphpad 6.0 statistical software (Graphpad, San Diego, CA, USA). Experimental data were collected and presented as mean ± standard deviation (SD). Statistical significance was analyzed using Student’s t tests and analysis of variance (ANOVA) with Tukey’s multiple comparisons test. The difference was significant when \( P < 0.05 \).

**Results**

**TPP attenuated PC12 cell injury induced by hypoxia**

The first set of experiments aimed to explore the possible roles of TPP on cell injury. The results, as shown in Figure 1(a), hypoxia induced decreasing
cell viability ($P < 0.01$), while TPP increased cell viability in hypoxia-stimulated cells ($P < 0.05$ or $P < 0.01$). Then, we further tested whether TPP revealed effects without hypoxia condition. Results showed that TPP at the concentration of 100, 200, and 300 $\mu$M has no effects on cell viability (Figure 1(b)). Therefore, TPP at 300 $\mu$M was chosen in the following experiments. In addition, the effects of TPP on hypoxia-treated cells apoptosis were detected. Results demonstrated that hypoxia enhanced cell apoptosis, while TPP alleviated cell apoptosis to some extent ($P < 0.05$; Figure 1(c)). Furthermore, hypoxia increased expression level of Bax ($P < 0.01$), cleaved Caspase-3 ($P < 0.001$), and cleaved PARP ($P < 0.001$) while decreased the expression of Bcl-2 ($P < 0.05$; Figure 1(d) and (e)), whereas TPP administration reversed the results induced by hypoxia (Figure 1(d) and (e)). In a word, TPP alleviated hypoxia-induced injury in PC12 cells.

**TPP induced downregulation of miR-34a**

Result demonstrated that expression of miR-34a was reinforced in the group treated with hypoxia ($P < 0.01$) while downregulated in the group

![Figure 1. Typhae pollen polysaccharides (TPP) alleviated hypoxia-induced injury in PC12 cells: (a) and (b) cell viability, (c) cell apoptosis, and (d) and (e) apoptotic proteins were detected by MTT assay, flow cytometry, and western blot, respectively. Data presented as mean ± standard deviation (SD) of three replicates. "HYPO" is short for "hypoxia" in the figure. *$P$ < 0.05; **$P$ < 0.01; and ***$P$ < 0.001 versus control were all significantly different; #$P$ < 0.05; ##$P$ < 0.01 versus hypoxia group were all significantly different.](image-url)
treated with TPP and hypoxia (P < 0.05; Figure 2). These results suggested that miR-34a might play a potential role in the regulation of TPP on hypoxia-induced cell injury.

TPP alleviated hypoxia-induced cell injury through silencing miR-34a

After transfection with miR-34a mimic and miR-34a inhibitor, the expression of miR-34a was examined by qRT-PCR. As shown in Figure 3(a), miR-34a mimic evidently enhanced miR-34a expression, while miR-34a inhibitor suppressed miR-34a expression, indicating highest transfection efficiency (P < 0.01). Then, we detected the role of miR-34a in the protective function by TPP in hypoxia-induced injured PC12 cell. Interestingly, the data showed that miR-34a overexpression brought in inhibition of cell viability (P < 0.05; Figure 3(b)) and enhancement of cell apoptosis in

Figure 2. Typhae pollen polysaccharides (TPP) downregulated expression of miR-34a. The expression of miR-34a was detected by qRT-PCR. Result was shown as mean ± standard deviation (SD) of three replicates. “HYPO” is short for “hypoxia” in the figure.

Figure 3. Typhae pollen polysaccharides (TPP) alleviated hypoxia-induced cell injury via downregulation of miR-34a: (a) miR-34a expression was detected by qRT-PCR, (b) cell viability, (c) cell apoptosis, and (d) and (e) cell apoptotic proteins were detected by MTT assay, flow cytometry, and western blot, respectively. Results were shown as mean ± standard deviation (SD) of three replicates. “HYPO” is short for “hypoxia” in the figure.

* P < 0.05; ** P < 0.01; and *** P < 0.001 versus control or negative control were all significantly different; # P < 0.05, ## P < 0.01 versus hypoxia group were all significantly different.
hypoxia and TPP-treated cells \((P < 0.01; \text{Figure } 3(\text{c}))\). Moreover, the apoptosis-related factors were also altered by miR-34a overexpression shown as decreasing Bcl-2 expression \((P < 0.05)\), increasing Bax \((P < 0.05)\), cleaved Caspase-3 \((P < 0.05)\), and cleaved PARP \((P < 0.01; \text{Figure } 3(\text{d}) \text{ and } (\text{e}))\). On the contrary, the enhancement of cell viability and the declination of cell apoptosis induced by TPP were overturned due to silencing miR-34a in hypoxia-disposed cells \((P < 0.05 \text{ or } P < 0.01; \text{Figure } 3(\text{b}) \text{ and } (\text{c}))\), which were further evidenced by the increase of Bcl-2 and the reduction of Bax, cleaved Caspase-3, and cleaved PARP \((P < 0.05 \text{ or } P < 0.01; \text{Figure } 3(\text{d}) \text{ and } (\text{e}))\). In summary, the above-mentioned results indicated that TPP promoted hypoxia-treated cell growth by downregulation of miR-34a.

**TPP promoted SIRT1 expression**

SIRT1 is closely associated with miR-34a.27 If the roles of TPP were realized via miR-34a, then whether SIRT1 was also related to TPP was explored. The expression of SIRT1 was determined. The results of qRT-PCR and western blot showed that hypoxia decreased SIRT1 expression, while TPP increased SIRT1 expression in hypoxia-treated cells \((P < 0.05 \text{ or } P < 0.01; \text{Figure } 4(\text{a}) \text{ and } (\text{b}))\). These data suggested that SIRT1 might also be involved in the effects of TPP in cell behavior.

**SIRT1 was a target of miR-34a**

Further experiments were performed to investigate the potential relationship between miR-34a and SIRT1. The expression of SIRT1 was downregulated by miR-34a overexpression compared with NC \((P < 0.01; \text{Figure } 5(\text{a}))\). Moreover, TargetScan (http://www.targetscan.org) were employed to predict target of miR-34a. The data revealed that miR-34a could directly bind to the 3'-UTR of SIRT1 mRNA (Figure 5(b)). For purpose of confirming this prediction, luciferase assay was carried out. As shown in Figure 5(c), the relative luciferase activity was decreased in the group co-transfection with SIRT1 wild-type plasmid and miR-34a mimic \((P < 0.01)\). However, there was no difference revealed in SIRT mutant groups (Figure 5(c)). These results indicated that SIRT1 was a target of miR-34a.

**TPP alleviated hypoxia-induced cell injury via upregulation of SIRT1**

To clarify the role which SIRT1 plays in the regulation system led by TPP in hypoxia-induced cell injury, si-SIRT1 was transfected into cells to decrease SIRT1 expression (Figure 6(a)). Similarly, the following experiments were performed. Results showed that silence of SIRT1 decreased cell viability \((P < 0.05; \text{Figure } 6(\text{b}))\), increased cell apoptosis \((P < 0.01; \text{Figure } 6(\text{c}))\), decreased expression of Bcl-2 \((P < 0.05)\), and upregulated expression of
Bax \((P < 0.05)\), cleaved Caspase-3 \((P < 0.01)\), and cleaved PARP \((P < 0.01)\) compared with NC (Figure 6(d) and (e)). Taken together, the consequences suggested that TPP alleviated hypoxia-induced cell injury through upregulation of SIRT1.

**TPP activated phosphatidylinositol 3′-kinase (PI3K)/protein kinase B (AKT) and Ras/Raf/MAP kinase–ERK kinase (MEK)/extracellular signal–regulated kinase (ERK) signal pathways through upregulation of SIRT1**

In our study, whether PI3K/AKT and Ras/Raf/MEK/ERK are involved in hypoxia-induced cell injury was also explored. As can be seen from Figure 7(a), hypoxia decreased the phosphorylation levels of PI3K \((P < 0.05)\) and AKT \((P < 0.01)\), while TPP reversed the results induced by hypoxia. Furthermore, silence of SIRT1 decreased the phosphorylation levels of PI3K \((P < 0.01)\) and AKT \((P < 0.05)\) as compared to NC, which indicated that SIRT1 silence broke the promoting effects of TPP on PI3K/AKT. On the contrary, hypoxia treatment downregulated the expression of Ras \((P < 0.05)\), Raf \((P < 0.01)\), phosphorylation of MEK \((P < 0.05)\), and ERK \((P < 0.05)\) while TPP led to the opposite results compared with control (Figure 7(b)). Similarly, silence of SIRT1 decreased the accumulated levels of Ras, Raf, phosphorylation of MEK and ERK. Taken together, these results showed that TPP activated PI3K/AKT and Ras/Raf/MEK/ERK signal pathway through upregulation of SIRT1.

**Discussion**

This study was performed to investigate the roles of TPP on hypoxia-treated cell viability and apoptosis. It is interesting to note that TPP alleviated hypoxia-induced cell injury as evidenced by increasing cell viability while decreasing apoptosis. Further experiments validated that the roles of TPP on hypoxia-treated cell growth were via downregulation of miR-34a. Moreover, SIRT1 was a target of mir-34a and TPP functions through upregulation of SIRT1. In the end, PI3K/AKT and Ras/Raf/MEK/ERK signal pathways are also involved in the regulation process.

An initial objective of the study was to verify the role of TPP on hypoxia-stimulated PC12 cells. Obviously, after hypoxia treatment, cell viability was decreased and cell apoptosis was enhanced, which indicated that hypoxia induced cell injury. As it is commonly evaluated, cell viability and
apoptosis are two vital aspects in the progression of cell growth. Our study demonstrated that TPP alleviated hypoxia-induced cell injury with evidence in increasing cell viability in a dose-dependent manner and decreased cell apoptosis. Previous study demonstrated TP functions in inhibiting autophagy in spinal cord injury of rats.28 This finding surprisingly noted that TPP, as one important component of TP, revealed anti-injury effects in PC12 cells.
In detail, the accumulated levels of apoptosis-related proteins were detected. Bax, cleaved Caspase-3, and cleaved PARP are pro-apoptotic proteins, while Bcl-2 is an anti-apoptotic protein. Results showed that hypoxia increased expression of Bax, cleaved Caspase-3, and cleaved PARP and decreased expression of Bcl-2. Differently, TPP administration reversed the expression trend, which could explain the finding in apoptosis. Previous study has proved that one of the important TP components typhae total flavone (PTF) blocked endoplasmic reticulum stress–induced cell apoptosis in human aortic vascular smooth muscle cells.31 This finding enriched the functions of TP on cell injury as it is corroborated that TPP, another TP component, also revealed anti-apoptosis roles in hypoxia-induced cell injury.

Very little was found in the literature on the regulation mechanism of about how TPP achieved its function in injury. Experiments were performed to explore further and deeper. It is well known that miRNAs were commonly involved in most of the diseases.32 For cerebral ischemia, there are various kinds of miRNAs involved in.9 MiR-34a is a tumor suppressor,33,34 but nowadays, it was revealed the role of miR-34a in a wide spectrum of diseases, such as in human placental diseases35 and coronary artery disease.36 Interestingly, overwhelming
findings demonstrated that miR-34a was related to cell apoptosis and cell cycle regulation. For example, activation of miR-34a was closely related to p53-mediated apoptosis.\textsuperscript{37,38} The results showed that TPP decreased miR-34a expression. Further experiments were performed to validate the inference that TPP might decrease cell apoptosis via downregulation of miR-34a. MiR-34a overexpression was achieved through transfection with miR-34a mimic. Interestingly, miR-34a overexpression decreased cell viability and increased apoptosis, which was in contrary to NC with TPP administration in hypoxia-treated PC12 cells. Similarly, miR-34a overexpression increased the accumulated levels of Bax, cleaved Caspase-3, and cleaved PARP while downregulated expression of Bcl-2. Therefore, it can be inferred that TPP achieved its function through downregulation of miR-34a. These results corroborated the effects of miR-34a on promoting cell apoptosis in a great deal of the previous works.\textsuperscript{37–39} Moreover, our finding enriched the roles of miR-34a associated not only with p53 but also with other apoptotic proteins Bax, cleaved Caspase-3, cleaved PARP, and Bcl-2.

In addition, a strong relationship between miR-34a and SIRT1 has been reported in the literature. Former literature pointed out that miR-34a works via an SIRT1-p53 pathway.\textsuperscript{40} To the beginning, results showed that TPP upregulated expression of SIRT1 which indicated that SIRT1 might be involved in the regulation of cell growth. Similarly, SIRT1 was a target of miR-34a, which was consistent with the previous report.\textsuperscript{41} Further experiments demonstrated that silence of SIRT1 reversed the results led by TPP, which suggested that TPP functions through miR-34a/SIRT1.

In the end, we investigated the potential signal pathways that were involved. PI3K/AKT and Ras/Raf/MEK/ERK signal pathways are closely associated with cerebral ischemia.\textsuperscript{52,43} Results showed that TPP activated PI3K/AKT and Ras/Raf/MEK/ERK signal pathways while silence of SIRT1 led to the opposite results, which hints that TPP modulated signal pathways via miR-34a/SIRT1. This finding broadly supports the work of other medicine, human, which showed its neuroprotective effect on cerebral ischemia by regulating PI3K/AKT pathway.\textsuperscript{44}

In a word, this study has proved the potential roles in regulating cell viability and apoptosis in hypoxia-treated PC12 cells via modulating miR-34a/SIRT1, as well as PI3K/AKT and Ras/Raf/MEK/ERK signal pathways. This study could provide a foundation for the treatment of cerebral ischemia in the future. The findings in this research are based on PC12 cell line. However, nerve growth factor–differentiated PC12 cells were not definitely determined which could affect the neuroprotective effect of TPP. Thus, the conclusions should be verified by results detected from more cell lines and in vivo model. Further studies are still required for the clinical trials to determine the potential roles of TPP on cerebral ischemia.

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