Thevetiaflavone from *Wikstroemia indica* ameliorates PC12 cells injury induced by OGD/R via improving ROS-mediated mitochondrial dysfunction

HUANKAI YAO[1], ZHENHAI YUAN[2], GUANGMING WEI[3], CHUN CHEN[1], JINGYU DUAN[1], YAN LI[1], YUNRU WANG[3], CHUNPING ZHANG[1] and YUANHUA LIU[3]

[1]School of Pharmacy and Jiangsu Key Laboratory of New Drug Research and Clinical Pharmacy, Xuzhou Medical University, Xuzhou, Jiangsu 221004; [2]Shandong Academy of Pharmaceutical Sciences, Jinan, Shandong 250101; [3]Department of Pharmacy, Taian City Central Hospital, Taian, Shandong 271000, P.R. China

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Abstract. Cerebral ischemia and following reperfusion affects many people worldwide. To discover efficient therapeutic approaches, numerous natural products have been investigated. The current study investigated the protective effects of thevetiaflavone, a natural flavonoid obtained from *Wikstroemia indica*, and the associated mechanisms using PC12 cells induced by oxygen and glucose deprivation. As a result, thevetiaflavone improves cell viability and suppresses the leakage of lactate dehydrogenase from the cytoplasm. Further investigation of the mechanisms demonstrated that thevetiaflavone decreases overproduction of ROS and ameliorates ROS-mediated mitochondrial dysfunction, including collapse of mitochondrial membrane potential and mitochondrial permeability transition pore opening. Thevetiaflavone reduces the intracellular Ca^{2+} level, which is closely associated with mitochondrial function and interplays with ROS. Furthermore, thevetiaflavone inhibits apoptosis in PC12 cells through upregulating the expression of Bcl-2 and downregulating that of Bax and caspase-3 in addition to increasing the activity of caspase-3. These results further indicate the protective effects of thevetiaflavone *in vivo* and its application in the clinic.

Correspondence to: Dr Chunping Zhang, School of Pharmacy and Jiangsu Key Laboratory of New Drug Research and Clinical Pharmacy, Xuzhou Medical University, 209 Tongshan Road, Xuzhou, Jiangsu 221004, P.R. China
E-mail: chunpingzhang520@163.com

Dr Yuanhua Liu, Department of Pharmacy, Taian City Central Hospital, 29 Longtan Road, Taian, Shandong 271000, P.R. China
E-mail: yhliuta@126.com

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Introduction

Cerebral stroke is a major cause of mortality and morbidity in adults around the world, and the global occurrence of stroke has markedly increased (1). Ischemic stroke accounts for about 87% of all stroke (2), and there are few effective therapeutic approaches for ischemic stroke (3). Though the pathology of ischemic stroke remains to be fully elucidated, several mechanisms including excitoxicity, oxidative stress, inflammation and apoptosis are involved (4). The interruption of blood flow in the brain will lead to the neuronal injury due to the oxygen and glucose deprivation (OGD), and timely reperfusion will exacerbate the damage due to the accumulation of reactive oxygen species (ROS) in mitochondria (5,6). This accumulation results in destruction of the mitochondria because the brain is susceptible to ROS (4). In addition, neurons in the penumbra will undergo mitochondrion-mediated apoptosis (5). In the discovery of novel therapeutic options for ischemic stroke, natural compounds serve a pivotal role, for example resveratrol (7), mangiferin (8) and kaempferide-7-O-(4''-O-acetylrhamnosyl)-3-O-rutinoside (9).

Wikstroemia indica is a medicinal plant belonging to the family of Thymelaeaceae and is distributed throughout northern China. In traditional Chinese medicine, it has been used for the treatment of arthritis, tuberculosis, syphilis and pertussis (10). Phytochemical investigations on this plant have identified that the major chemical constituents include flavonoids (11-13), lignans (14,15) and coumarins (16,17). The current study aimed to investigate bioactive phytochemicals for the prevention of ischemic stroke by determining the chemical constituents from the roots of *Wikstroemia indica* and examining their neuroprotective effects. In the current study, it was reported that thevetiaflavone isolated from *Wikstroemia indica* demonstrated protective effects on PC12 cells induced by oxygen and glucose deprivation/reoxygenation (OGD/R) and relevant mechanisms.

Materials and methods

Chemicals and reagents. Thiazolyl blue tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from

Shanghai Aladin Biochemal Technology Co., Ltd. (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) was obtained from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). ROS, lactate dehydrogenase (LDH) and bicinchoninic acid (BCA) assay kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). MitoProbe transition pore assay kit was from Thermo Fisher Scientific, Inc.. Rhodamine-123, Fluo-3 AM and caspase-3 assay kits in addition to cleaved caspase-3, Bcl-2, Bax and β-actin antibodies were purchased from Beyotime Institute of Biotechnology (Nantong, China).

Isolation of thevetiaflavone. The roots of *Wikstroemia indica* (5.0 kg) were collected in July 2011 in Luofu Mountain (China), and were identified by one of the authors, Dr Chunping Zhang. The voucher specimen (Y20110701) was deposited in Xuzhou Medical University.

The air dried roots of *Wikstroemia indica* were ground and extracted with petroleum ether and acetone successively. The acetone extract (240 g) was repeatedly subjected to common column on silica gel with gradient dichloromethane/methnol (from 100:0 to 70:30) and yielded a yellowish powder (18.0 mg). The structure of this compound was determined as thevetiaflavone by analysis of ^{1}H and ^{13}C NMR spectra in addition to comparison with the data reported (18).

Cell culture and treatment. Rat adrenal pheochromocytoma cells (PC12 cells), the neuron-like cells, were obtained from Cell Bank of Shanghai Institute of Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM containing 10% fetal bovine serum, 1% penicillin and streptomycin at 37˚C in a humid atmosphere with 5% CO_2 and 95% air. Then the OGD/R was performed as described previously (19). Briefly, subsequent to the normal medium being replaced by glucose-free DMEM, the cells were cultured under a 95% N_2 and 5% CO_2 atmosphere at 37˚C in the presence or absence of certain concentrations of thevetiaflavone (0.1, 1 and 10 μM) for 4 h. Subsequently, the cells were cultured again under normal conditions (5% CO_2 and 95% air) for 24 h with or without thevetiaflavone. The cells in the control group were cultured at a normal atmosphere.

Cell viability assay. The PC12 cell viability was evaluated through the MTT assay. The cells were cultivated in 96-well microplates at $1x10^5$ per well. After treatment as above, MTT (0.5 mg/ml) was added into each well and the mixture was incubated for 4 h at 37˚C. Subsequently, the medium was removed and the formazan was dissolved in DMSO. After 10 min, the optical density (OD) was recorded on a microplate reader at 550 nm. The cell viability was expressed as the relative percentage of OD values compared with the control group.

Extracellular LDH activity. For the leakage of intracellular LDH is associated with the cell survival, the extracellular LDH activity in cells medium was measured by the LDH assay kit. Following treatment, the supernatant (20 μl) in each well was collected and tested following the manufacturer's instructions. Thereafter the OD values were recorded on a microplate reader at 450 nm. The activity extracellular LDH was derived from the OD values as U/l.

Level of intracellular ROS. For the excessive production of ROS induced by OGD/R will promote the injury, the level of intracellular ROS was assayed by an assay kit following the manufacturer's instructions. Following treatment, the cells were rinsed with PBS and 10 μM DCFH-DA solution was added. Then the incubation was implemented for 30 min at 37˚C. The cells were washed with PBS and then the fluorescence intensity of hydrolyzed DCFH-DA was determined by a fluorescence spectrophotometer at excitation wavelength of 485 nm and emission wavelength of 520 nm.

Intracellular Ca^{2+} level. Increasing ROS will cause the overload of intracellular Ca^{2+} through activating signaling transduction cascades. High levels of Ca^{2+} will result in the overproduction of ROS (20), and in order to determine the intracellular Ca^{2+} level, the fluorescence probe Fluo-3 AM has been used previously (7). The treated PC12 cells were washed with PBS three times, and incubated with Fluo-3 AM at 37˚C for 30 min in the dark. Then the cells were washed with PBS to remove excessive extracellular dye. The fluorescence intensity was immediately detected on a fluorescence spectrophotometer at 488 nm for excitation and 525 nm for emission.

Mitochondrial membrane potential (MMP). MMP of PC12 cells was quantified by a fluorescence probe rhodamine-123, which accumulates in mitochondria of living cells. However if there is a collapse of MMP, rhodamine-123 will release from mitochondria due to the membrane depolarization. The treated PC12 cells were incubated with 2 μM rhodamine-123 for 15 min. Subsequently, the fluorescence intensity was recorded on a fluorescence spectrophotometer and the excitation wavelength in addition, emission wavelength was set at 488 nm and 525 nm respectively. The MMP level was expressed as the ratio compared with the control group.

Mitochondrial permeability transition pore (mPTP) opening. To further investigate the function of mitochondria in PC12 cells induced by OGD/R, mPTP opening was detected by the calcein-AM loading/Co^{2+} quenching technique through a MitoProbe transition pore assay kit following the manufacturer's instructions. The non-fluorescent calcein-AM can freely pass through the cellular membrane to be hydrolyzed by intracellular esterases as fluorescent calcein, which is captured inside cells. The loading of Co^{2+} can quench the intracellular fluorescence of calcein except that in mitochondria, for Co^{2+} cannot enter mitochondria. Therefore, when mPTP opens, the fluorescence of calcein in mitochondria will be quenched by Co^{2+} (21). The PC12 cells were pre-treated as above, and washed with PBS for three times. Then the cells were suspended in Hanks' balanced salt solution supplemented with 2 mM Ca^{2+} (HBSS/Ca^{2+}), and incubated with 2 μM Calcein-AM and 1 mM $CoCl_2$ at 37˚C for 30 min in dark. Subsequently to washing with HBSS to remove the excessive reagents, the cells were resuspended in HBSS/Ca^{2+}. The fluorescence intensity of calcein was directly detected on a fluorescence spectrophotometer at 494 nm for excitation and 517 nm for emission, and at least $1x10^4$ events per sample were acquired.

Intracellular ATP level. The intercellular ATP was determined by a firefly luciferase method according to the manufacturer's

protocol of the assay kit. ATP is consumed quantitatively when the luciferin emits fluorescence under the catalysis of firefly luciferase. The treated PC12 cells were lysed on ice with 200 μl lysis solution from the assay kit. Then the lysate was centrifuged at 12,000 x g for 4 min at 4˚C. Thereafter supernatant (100 μl) was aspirated for the quantification and 100 μl ATP monitoring reagent was added. The luminescence was detected on the fluorescence spectrophotometer. The level of intracellular ATP was calculated from the standard curve and expressed as the percentage associated with the control group.

Caspase-3 activity. The activity of Caspase-3 in PC12 cells was tested by a colorimetric assay kit according to the manufacturer's instruction. Following treatment, the cells were washed with PBS. Then the cells were lysed on ice and centrifuged at 16,000 x g and 4˚C for 10 min. The supernatant was sucked and incubated with substrate (Ac-DEVD-*p*NA) at 37˚C for 2 h. The OD values were recorded on a microplate reader at 405 nm. The relative activity of caspase-3 was expressed as the OD values of the product *p*NA produced by caspase-3.

Western blot analysis. The treated PC12 cells were subjected to western blot analysis for the expression of caspase-3, Bcl-2, and Bax. The cells were lysed on ice for 30 min with lysis solution containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton and 1 mM phenylmethylsulfonyl fluoride. Then the lysate was centrifuged at 10,000 x g for 15 min at 4˚C, and the supernatant was collected as the total protein for the following analysis. The total protein was determined by a BCA assay kit, and subsequently separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Then the samples were transferred to a polyvinylidene fluoride membranes. The membranes were blocked with nonfat milk at room temperature, and then incubated overnight with primary antibodies of cleaved caspase-3 (cat no. AC033; 1:1,000), Bcl-2 (cat no. AB112; 1:1,000), Bax (cat no. AB026; 1:1,000) and β-actin (cat no. AF0003; 1:1,000) at 4˚C. After washing three times with buffer solution including 0.1% Tween-20, the membranes were incubated with the corresponding secondary antibody (cat no. A1092; 1:1,000) conjugated with horseradish peroxidase in blocking solution at room temperature for 1 h and detected by enzyme-linked chemiluminescence substrate. β-actin was used as the internal control.

Statistical analysis. All data were expressed as the mean ± standard deviation and analyzed by GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Data among different groups were analyzed by one way analysis of variance followed by Dunnett test for multiple comparisons and Student's t-test for single comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Phytochemical investigation. The structure of thevetiaflavone can be elucidated by analysis of ^{1}H and ^{13}C NMR spectra. In ^{1}H NMR spectra, δ_H 6.38 (1H, d, *J*=2.2 Hz) and 6.78 (1H, d, *J*=2.2 Hz) are proton signals in an AX system, which indicate there is a benzene with two hydrogen atoms in meta position. δ_H 7.96 (2H, d, *J*=8.8 Hz) and 6.93 (2H, d, *J*=8.8 Hz) are proton signals in an A_2X_2 system, which belong to the benzene ring with substitution in para position. In addition, proton signal of methoxyl hydrogen atoms can be detected at δ_H 3.87 (3H, s). In ^{13}C NMR spectrum, there are 15 carbon signals together with the carbon signal of methoxyl group at δ_C 56.2. These data are in accordance with our former report (18), the structure of this compound can be identified as thevetiaflavone (Fig. 1). The ^{1}H and ^{13}C NMR data were assigned as following: ^{1}H NMR (DMSO-d_6) δ: 6.86 (1H, s, H-3), 6.38 (1H, d, *J*=2.2 Hz, H-6), 6.78 (1H, d, *J*=2.2 Hz, H-8), 7.96 (2H, d, *J*=8.8 Hz, H-2', 6'), 6.93 (2H, d, *J*=8.8 Hz, H-3', 5'), 3.87 (3H, s, -OCH_3); ^{13}C NMR (DMSO-d_6) δ: 164.2 (C-2), 103.2 (C-3), 182.1 (C-4), 157.4 (C-5), 98.1 (C-6), 165.3 (C-7), 92.8 (C-8), 161.3 (C-9), 104.8 (C-10), 121.2 (C-1'), 128.7 (C-2', 6'), 114.4 (C-3', 5'), 161.5 (C-4'), 56.2 (-OCH_3).

Effect of thevetiaflavone on the viability of PC12 cells induced by OGD/R. As presented in Fig. 2, treatment by OGD/R could result in the poor viability of PC12 cells (52.93±3.87%, P<0.001) compared with the control group. When pre-treated with thevetiaflavone, the survival of PC12 cells was apparently improved in a dosage-dependent manner (59.37±5.70, 65.72±6.23 and 77.89±7.48%), which indicated the protective effect of thevetiaflavone on PC12 cells injured by OGD/R.

Effect of thevetiaflavone on the release of LDH. To evaluate the protective effects of thevetiaflavone, the extracellular LDH activity released was evaluated. Consequently, OGD/R can significantly increase the activity of extracellular LDH as 84.27±6.29 U/l compared with the control group (42.06±4.37 U/l, P<0.001 vs. control group). In contrast, in the presence of thevetiaflavone, the activity will be reduced as 77.42±2.55, 67.59±5.29 and 62.20±5.03 U/l, respectively (Fig. 3). These results further validate thevetiaflavone can improve the viability of PC12 cells injured by OGD/R.

Effect of thevetiaflavone on ROS production in PC12 cells induced by OGD/R. The production of intracellular ROS was detected. As a result, OGD/R can lead to the overproduction of intracellular ROS (325.49±15.33, P<0.001 vs. the control group) and thevetiaflavone can clearly suppress this overproduction (289.15±21.72, 212.46±28.74 and 190.44±24.47) (Fig. 4).

Effect of thevetiaflavone on intracellular Ca^{2+} in PC12 cells induced by OGD/R. As presented in Fig. 5, the intracellular Ca^{2+} level was sharply elevated by OGD/R (780.01±34.28, P<0.001 vs. control group). And with different concentrations of thevetiaflavone, the levels of intracellular Ca^{2+} were significantly reduced as 712.01±65.03, 614.23±410.14 and 542.14±60.14.

Effect of thevetiaflavone on MMP and mPTP in PC12 cells induced by OGD/R. Herein, we have found OGD/R can damage the function of mitochondria in PC12 cells including decreasing MMP (36.22±7.15%, P<0.001 vs. control group) and increasing the mPTP opening (48.44±4.67%, P<0.001 vs. control group). In the presence of thevetiaflavone, the collapse of MMP was attenuated (44.50±4.88%, 58.42±6.46 and 78.89±6.02%; Fig. 6), and blocking of the mPTP opening

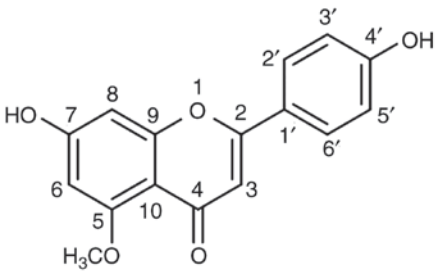

Figure 1. Chemical structure of thevetiaflavone.

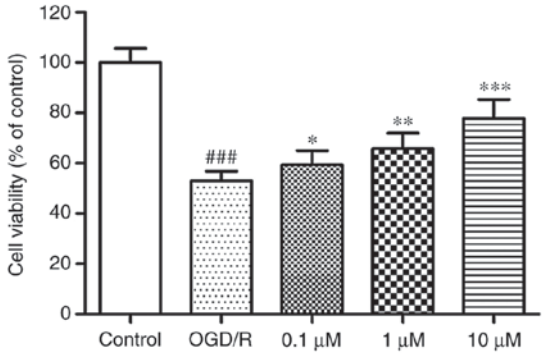

Figure 2. Effect of thevetiaflavone on cell viability in PC12 cells induced by OGD/R. Data are presented as the mean ± standard deviation (n=6, ###P<0.001 vs. control group, *P<0.05, **P<0.01 and ***P<0.001 vs. OGD/R group). OGD/R, oxygen and glucose deprivation/reoxygenation.

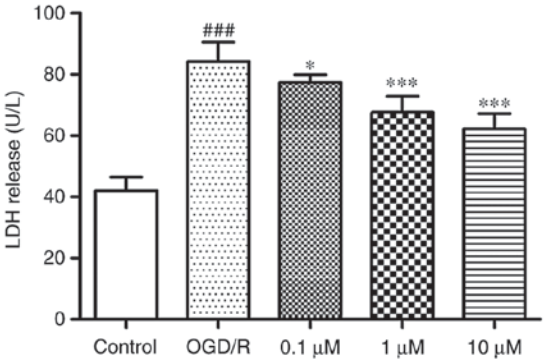

Figure 3. Effect of thevetiaflavone on the release LDH in PC12 cells induced by OGD/R. Data are presented as the mean ± standard deviation (n=6, ###P<0.001 vs. control group, *P<0.05 and ***P<0.001 vs. OGD/R group). LDH, lactate dehydrogenase; OGD/R, oxygen and glucose deprivation/reoxygenation.

was observed (58.16±6.74%, 63.42±8.26% and 81.40±4.45%; Fig. 7). These results demonstrate the protective effects of thevetiaflavone on dysfunction of mitochondria in PC12 cells.

Effect of thevetiaflavone on ATP level in PC12 cells induced by OGD/R. The detection of intracellular ATP was further detected to confirm the protective effects of thevetiaflavone. As a result, thevetiaflavone can promote ATP production in a dosage-dependent manner (63.54±6.83, 71.89±7.33 and 79.39±7.64%), though OGD/R has induced the decline of ATP production (53.93±6.30%, P<0.001 vs control group) in PC12 cells (Fig. 8).

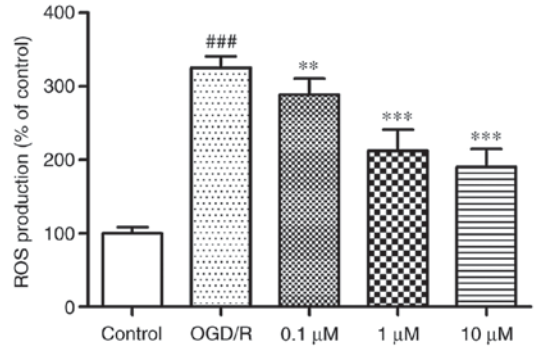

Figure 4. Effect of thevetiaflavone on ROS production in PC12 cells induced by OGD/R. Data are presented as the mean ± standard deviation (n=6, ###P<0.001 vs. control group, **P<0.01 and ***P<0.001 vs. OGD/R group). ROS, reactive oxygen species; OGD/R, oxygen and glucose deprivation/reoxygenation.

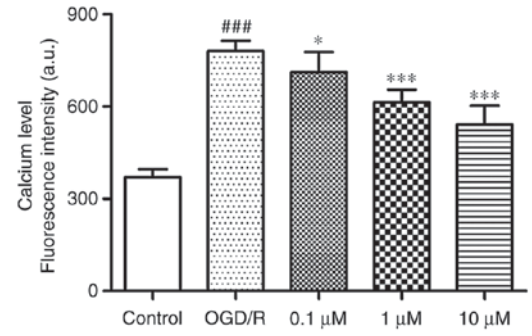

Figure 5. Effect of thevetiaflavone on intracellular calcium level in PC12 cells induced by OGD/R. Data are presented as the mean ± standard deviation (n=6, ###P<0.001 vs. control group, *P<0.05 and ***P<0.001 vs. OGD/R group). OGD/R, oxygen and glucose deprivation/reoxygenation.

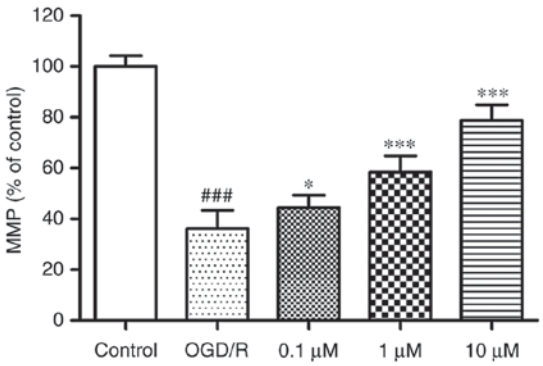

Figure 6. Effect of thevetiaflavone on MMP in PC12 cells induced by OGD/R. Data are presented as the mean ± standard deviation (n=6, ###P<0.001 vs. control group, *P<0.05 and ***P<0.001 vs. OGD/R group). MMP, mitochondrial membrane potential; OGD/R, oxygen and glucose deprivation/reoxygenation.

Effect of thevetiaflavone on apoptosis of PC12 cells induced by OGD/R. To evaluate the protective effect of thevetiaflavone on PC12 cells induced by OGD/R against apoptosis, western blot analysis for caspase-3, Bcl-2 and Bax was implemented. In that analysis, following induction by OGD/R, the expression of Bcl-2 in PC12 cells was downregulated, whilst caspase-3 and Bax were upregulated. By contrast, with the pre-treatment of thevetiaflavone, the expression of Bcl-2 was upregulated and

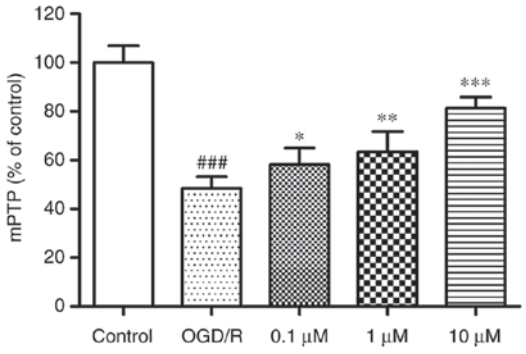

Figure 7. Effect of thevetiaflavone on mPTP in PC12 cells induced by OGD/R. Data are presented as the mean ± standard deviation (n=6, ###P<0.001 vs. control group, *P<0.05, **P<0.01 and ***P<0.001 vs. OGD/R group). mPTP, mitochondrial permeability transition pore; OGD/R, oxygen and glucose deprivation/reoxygenation.

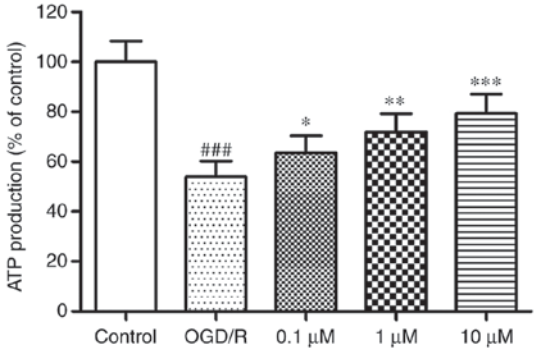

Figure 8. Effect of thevetiaflavone on ATP production in PC12 cells induced by OGD/R. Data are presented as the mean ± standard deviation (n=6, ###P<0.001 vs. control group, *P<0.05, **P<0.01 and ***P<0.001 vs. OGD/R group). ATP, adenosine triphosphate; OGD/R, oxygen and glucose deprivation/reoxygenation.

caspase-3 together with Bax was downregulated (Fig. 9A) at the same time, the determination of caspase-3 activity further indicated thevetiaflavone could attenuate PC12 cells apoptosis induced by OGD/R (0.76±0.05, 0.68±0.07 and 0.57±0.06) by comparison with OGD/R group (0.82±0.05) (Fig. 9B). These results elucidated the anti-apoptotic effects of thevetiaflavone on PC12 cells induced by OGD/R.

Discussion

Cerebral ischemia and reperfusion injury is caused by OGD/R following the interruption of blood and results in infarction. Around the infarct area, the penumbral area can be attenuated through the inhibition of apoptosis (5). The activity of extracellular LDH is closely associated with cell viability, due to the fact that as a stable cytoplasmic enzyme in cells, LDH can be rapidly released into the culture medium when the plasma membrane is damaged (22). In our investigation, thevetiaflavone can attenuate the viability of PC12 cells injured by OGD/R. Further investigation on thevetiaflavone has indicated that it can reduce the increased extracellular LDH activity induced by OGD/R, which markedly confirmed the protective effects of thevetiaflavone on the injured PC12 cells.

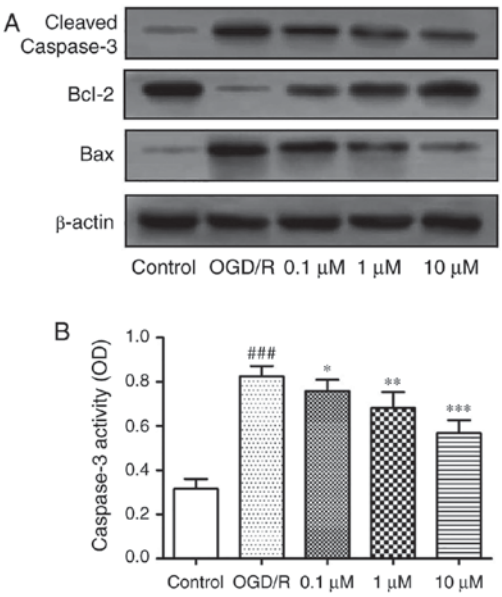

Figure 9. Effect of thevetiaflavone on cell apoptosis in PC12 cells induced by OGD/R. (A) Western blot analysis for proteins associated with apoptosis including caspase-3, Bcl-2 and Bax. (B) Caspase-3 activity affected by thevetiaflavone. Data are presented as the mean ± standard deviation (n=6, ###P<0.001 vs. control group, *P<0.05, **P<0.01 and ***P<0.001 vs. OGD/R group). OGD/R, oxygen and glucose deprivation/reoxygenation; OD, optical density.

In cerebral ischemia and reperfusion, the neurons in the penumbra will undergo mitochondrion-mediated apoptosis without the improvement of ROS-induced mitochondrial dysfunction (5). Excessive ROS can interplay with Ca^{2+} and result in overload of Ca^{2+}. The increasing intracellular Ca^{2+} will affect redox balance in turn to generate more ROS (20,23). As the major site of ROS production, mitochondria are both targets and sources of oxidative stress and susceptible to ROS (24). Oxidative stress is an activator of apoptosis through oxidative damage of the mitochondrial membrane proteins and lipids, which results in the dysfunction of mitochondria (25). In addition, the Ca^{2+} serves a central role in regulating mPTP opening through affecting ATP/ADP balance by tricarboxylic acid cycle, electron transport chain, and MMP (26). In the presence of thevetiaflavone, the mitochondrial dysfunction including overproduction of ROS, overload of Ca^{2+}, collapse of MMP, mPTP opening and declined ATP was improved in PC12 cells induced by OGD/R, which demonstrated thevetiaflavone could ameliorate the mitochondrial dysfunction.

As a member of cysteine-dependent aspartate proteases family, caspase-3 is one of the effector enzymes in apoptosis and activated through cleavage in ischemia (27). Bcl-2 and Bax are members of the Bcl-2 protein family and involved in the mitochondrion-drove apoptosis through oligomerization (25). Bcl-2 displays anti-apoptotic effects and can attenuate the activation of caspase-3 while Bax exhibits pro-apoptotic effects (28). In PC12 cells, the expression of Bcl-2 was downregulated, whilst caspase-3 and Bax was upregulated following the treatment of OGD/R, which indicated apoptosis occurred herein. In contrast, with thevetiaflavone, the expression of Bcl-2 was upregulated, whilst caspase-3 and Bax were downregulated. The quantitative detection of caspase-3 activity also obtained consistent results. These results indicate that

thevetiaflavone can relieve the apoptosis induced by OGD/R in PC12 cells.

In summary, the current study has evaluated the protective effects of thevetiaflavone on PC12 cells injury induced by OGD/R. It is suggested that the mechanism is associated with the improvement of ROS-mediated mitochondrial dysfunction. These results provide a basis to further investigate the effects *in vivo* and suggests a potential application in clinical practice.

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