Research Paper

Effect of Evodiamine on 6-Hydroxydopamine Induced Damage of Parkinson's Disease Cell Model by Modulating MicroRNA-9-5p

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To explore the effect of evodiamine on Parkinson's disease and its possible mechanism. 6-hydroxydopamine challenged SK-N-SH cells were adopted to mimic Parkinson's disease condition *in vitro*. Levels of proteins and genes were tested using Western blot and quantitative reverse transcription-polymerase chain reaction. Oxidative stress was evaluated by detecting the expression of lactate dehydrogenase and glutathione. Enzyme-linked immunosorbent assay analysis and flow cytometry were applied for the examination of inflammatory reaction and apoptosis rate. Evodiamine treatment could dose-dependently inhibit lactate dehydrogenase activity and cell apoptosis, reduced the levels of inflammatory factors, but elevated glutathione content in 6-hydroxydopamine-challenged SK-N-SH cells. Evodiamine exposure led to the decline of microRNA-9-5p content. Inhibition of microRNA-9-5p was able to protect against 6-hydroxydopamine stimulated oxidative stress, elevation of apoptosis and inflammatory factors in cells. Besides that, boosting microRNA-9-5p attenuated the protective functions of evodiamine on 6-hydroxydopamine stimulated SK-N-SH cells. Evodiamine could inhibit 6-hydroxydopamine induced oxidative, inflammatory and apoptotic injury in the Parkinson's disease cell model *via* microRNA-9-5p.

Key words: Evodiamine, Parkinson's disease, microRNA-9-5p, 6-hydroxydopamine, inflammation

As one of common neurodegenerative diseases in clinic, Parkinson's Disease (PD) clinical manifestations are cognitive dysfunction, sensory impairment, etc. It has been revealed that neuron apoptosis, oxidative stress and neuroinflammation have significant roles in the progression of PD[1-3], thus, probing the PD pathogenesis is urgent for progressing new therapeutic targets for PD.

Currently, studies have shown the important role of Traditional Chinese Medicine (TCM) in disease treatment, as the indispensable part of TCM, Herbal Medicine (HM) possesses antioxidant and anti-inflammatory effects, and can be used to reduce nerve cell damage and treat PD[4-6]. The evodiamine is a frequently used HM and has been revealed to have protective effects on cardiovascular and nervous system, as well as anti-inflammatory and antioxidant effects and can be used to treat tumors, Alzheimer's disease, dysentery, cardiovascular disorders and antimicrobials in clinical practice[7-10]. However, there are relatively few studies on the relationship between evodiamine and PD. Micro Ribonucleic Acids (miRNAs) have been uncovered to participate in many aspects of cell biology[11,12], among which, it was discovered that PD cell model showed higher miR-9-5p level and its down-regulation devastated neuronal apoptosis, oxidative stress and inflammation[13,14]. However, it is not known whether evodiamine can affect PD process *via* modulating miR-9-5p.

Therefore, this study adopted 6-Hydroxydopamine (6-OHDA) challenged human SK-N-SH neuroblastoma cell lines to establish a cell model of PD, then whether evodiamine could affect the PD process *via* miR-9-5p was explored.

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MATERIALS AND METHODS

Cell culture and treatment:

SK-N-SH cell lines were purchased from American Type Culture Collection (ATCC) (Manassas, Virginia, United States of America (USA)). The high glucose Dulbecco's Modified Eagle Medium (DMEM) (ATCC) with 1 % penicillin/streptomycin and 10 % Fetal Bovine Serum (FBS) (Solarbio, Shanghai, China) were applied to culture SK-N-SH cells. The medium was replaced every day. Then SH-SY5Y cells were differentiated to have the biological characteristics of dopamine neurons using vitamin A and Type Plasminogen Activator (TPA) (Life Technologies)[15]. SK-N-SH cells cultured with completed DMEM medium were deemed to be the control group. In functional experiments, cells cultured with 100 µmol/l 6-OHDA in completed DMEM medium was utilized to induce PD model cells (PD group). In addition, different concentrations evodiamine (1 µmol/l, 5 µmol/l or 10 µmol/l) was applied to incubate with SK-N-SH cells overnight, which were reacted with 6-OHDA (100 µmol/l) overnight for another analysis.

Cell transfection:

MiR-9-5p mimic (miR-9-5p) and the inhibitor (anti-miR-9-5p) with the controls (miR-NC or anti-miR-NC) were provided by Genema (Shanghai, China), the transient transfection was performed adopting Lipofectamine 2000.

Detection of Lactate Dehydrogenase (LDH) and Glutathione (GSH):

The culture of SK-N-SH cells was gathered, then active of LDH were tested using 2,4-Dinitrophenylhydrazine test. Besides, cells in different groups were lysed by repeated freeze-thaw cracking, then the supernatant was gathered and GSH content was tested by the GSH Peroxidase assay kit.

Enzyme-Linked Immunosorbent Assay (ELISA):

ELISA Kits (Abcam, Cambridge, Massachusetts) were adopted to test levels of inflammatory cytokines. SK-N-SH cell supernatant were gathered by centrifugation, after the reaction with antibody cocktail, substrate and Stop solution, the absorption was read by a microplate reader.

Flow cytometry:

After indicated treatment, 500 µl binding buffer suspended cells were added into the cell precipitation. Then Annexin V-Fluorescein Isothiocyanate (FITC) (5 µl) and Propidium Iodide (PI) (5 µl) were added respectively according to the instructions of the apoptosis kit, followed by a flow cytometry analysis.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR):

After the isolation of total RNA *via* the Trizol reagent (Beyotime, Shanghai, China), reverse transcription was performed for complementary Deoxyribonucleic Acid (cDNA) generation, and qRT-PCR amplification using cDNA as template was conducted. The content of miR-9-5p expression was calculated through the Cycle threshold (Ct) value with U6 as the reference control.

Western blot:

The total protein was extracted by Radioimmunoprecipitation Assay (RIPA) buffer (Beyotime). After being transferred into Polyvinylidene Difluoride (PVDF) membrane[16], the protein was enclosed with 5 % skim milk for 2 h at 37°. The diluent of B-cell lymphoma 2 (Bcl-2) (1:1000) and Bcl-2-Associated X Protein (BAX) (1:1000) primary antibody and the diluent of internal reference Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) (1:2000) were added and incubated at 4° for 12 h. The diluent of the second antibodies was added (1:3000) and incubated at 37° for 1 h. ImageJ software to analyze the gray value of each band after analyzing by Electrochemiluminescence (ECL) system.

Statistical analysis:

The data were manifested as ($\bar{x}\pm s$). The comparison between groups were executed using Statistical Package for the Social Sciences (SPSS) 21.0 statistical software with the t-test or analysis of variance. $p<0.05$ indicated statistically significant.

RESULTS AND DISCUSSION

Compared with the untreated SK-N-SH cells, 6-OHDA treatment markedly increased LDH active and the content of Interleukin (IL)-1 beta (β), IL-6 and IL-18, but decreased GSH content in SK-N-SH cells (Table 1). Besides that,

evodiamine treatment dose-dependently reduced the contents of LDH, IL-1β, IL-6 and IL-18, and increased GSH level in SK-N-SH cells treated with 6-OHDA as shown in Table 1

As shown in fig. 1, 6-OHDA treatment induced apoptosis, accompanied with the declined Bcl-2 protein and elevated BAX protein in SK-N-SH cells (fig. 1A, fig. 1B and Table 2). In addition, evodiamine treatment could dose-dependently suppress SK-N-SH cell apoptosis and increased Bcl-2 protein as well as decreased BAX protein under 6-OHDA stimulation as shown in fig. 1A and fig. 1B.

Levels of miR-9-5p in the PD group was increased relative to control group. Moreover, miR-9-5p content was decreased after 1 µmol/l, 5 µmol/l and 10 µmol/l evodiamine treatment as shown in Table 3.

The 6-OHDA induced SK-N-SH cells in anti-miR-9-5p group exhibited reduced LDH activity, levels of IL-1β, IL-6 and IL-18, apoptosis rate and BAX protein, but elevated GSH content and Bcl-2 protein level relative to cells in anti-miR-NC group as shown in fig. 2A, fig. 2B and Table 4.

Compared with the 10 µmol/l+miR-NC group, the expression level of miR-9-5p, the LDH activity and the levels of IL-1β, IL-6 and IL-18 in the 10 µmol/l+miR-9-5p group were increased as shown in Table 5.

Relative to the 10 µmol/l+miR-NC group, the apoptosis of 6-OHDA induced SK-N-SH cells were reduced in the 10 µmol/l+miR-9-5p group, accompanied with the increased BAX and decreased Bcl-2 protein as shown in fig. 3A, fig. 3B and Table 6.

TABLE 1: EFFECTS OF EVODIAMINE ON THE OXIDATIVE STRESS AND INFLAMMATION OF 6-OHDA CHALLENGED SK-N-SH CELLS (x̄±s, n=9)

Group	LDH (U/l)	GSH (mg/l)	IL-1β (pg/ml)	IL-6 (pg/ml)	IL-18 (pg/ml)
Control	13.36±1.27	87.40±3.61	2.80±0.57	8.68±0.49	9.81±0.78
PD	58.85±4.60[a]	33.83±3.18[a]	23.83±2.31[a]	37.80±3.41[a]	49.88±4.00[a]
1 µmol/L EVO	42.00±2.07[b]	56.03±1.68[b]	17.68±0.54[b]	28.85±1.30[b]	32.92±2.54[b]
5 µmol/L EVO	24.13±4.86[bc]	65.31±2.97[bc]	15.11±0.77[bc]	22.62±2.40[bc]	21.26±2.38[bc]
10 µmol/L EVO	18.12±0.65[bcd]	78.89±1.40[bcd]	10.03±0.40[bcd]	14.55±1.81[bcd]	13.79±1.01[bcd]
F	312.724	534.005	423.7	263.208	397.402
p	0	0	0	0	0

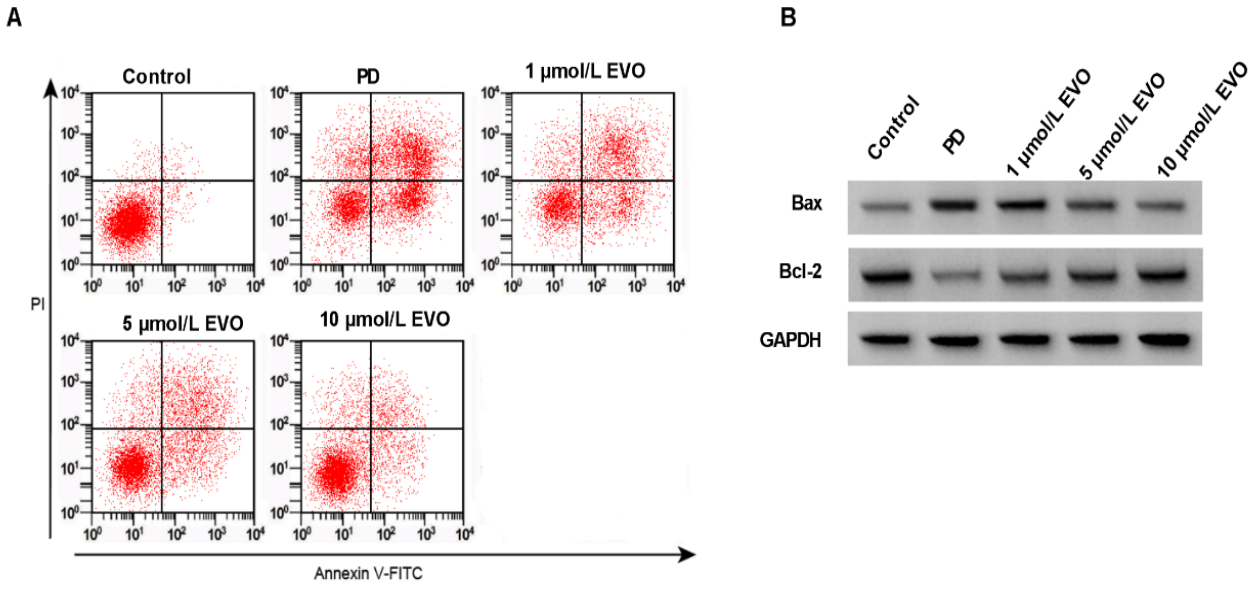

Fig. 1: Effects of evodiamine on the apoptosis of 6-OHDA induced SK-N-SH cells, (A): Cell apoptosis analysis by flow cytometry and (B): Western blot for the levels of Bcl-2 and BAX proteins

TABLE 2: EFFECTS OF EVODIAMINE ON THE APOPTOSIS OF 6-OHDA INDUCED SK-N-SH CELLS (x̄±s, n=9)

Group	Apoptosis rate (%)	BAX	Bcl-2
Control	6.10±1.14	0.24±0.02	0.93±0.05
PD	30.93±3.64[a]	0.85±0.03[a]	0.27±0.05[a]
1 μmol/L EVO	20.11±1.44[b]	0.59±0.11[b]	0.50±0.04[b]
5 μmol/L EVO	14.98±0.71[bc]	0.44±0.03[bc]	0.67±0.03[bc]
10 μmol/L EVO	10.94±0.64[bcd]	0.33±0.02[bcd]	0.79±0.03[bcd]
F	232.601	176.173	353.143
p	0	0	0

TABLE 3: THE EFFECTS OF EVODIAMINE ON miR-9-5P CONTENT IN 6-OHDA CHALLENGED SK-N-SH CELLS (x̄±s, n=9)

Group	miR-9-5p
Control	1.00±0.05
PD	4.53±0.22[a]
1 μmol/l EVO	3.15±0.10[b]
5 μmol/l EVO	2.35±0.15[bc]
10 μmol/l EVO	1.25±0.05[bcd]
F	1096.02
p	0

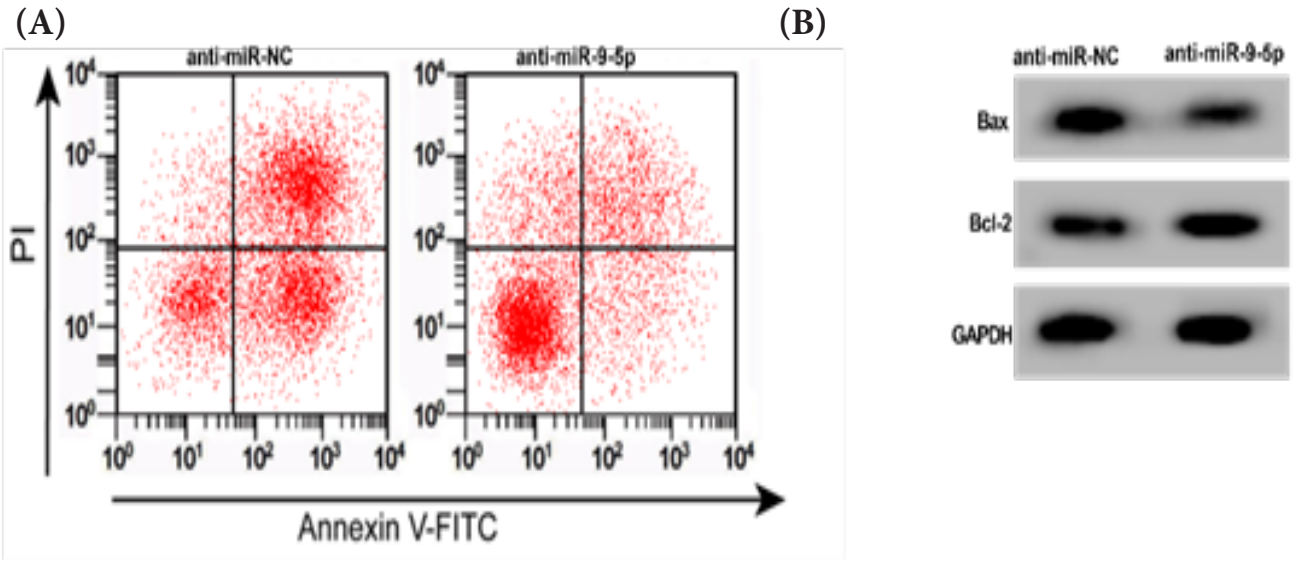

Fig. 2: Effects of miR-9-5p inhibition on the apoptosis of 6-OHDA challenged SK-N-SH cells, (A): Analysis of cell apoptosis *via* flow cytometry and (B): Measurement of Bcl-2 and BAX proteins *via* Western blot

TABLE 4: THE EFFECTS OF miR-9-5P INHIBITION ON 6-OHDA INDUCED SK-N-SH CELLS

Group	miR-9-5p	LDH (U/l)	GSH (mg/l)	IL-1β (pg/ml)	IL-6 (pg/ml)	IL-18 (pg/ml)	Apoptosis rate (%)	BAX	Bcl-2
Anti-miR-NC	1.00±0.07	58.14±5.08	33.55±5.68	23.90±2.88	37.96±1.24	49.45±5.19	30.37±2.06	0.86±0.04	0.27±0.03
Anti-miR-9-5p	0.27±0.04[a]	24.31±2.34[a]	65.34±2.03[a]	15.06±0.93[a]	22.57±1.85[a]	21.88±2.04[a]	14.63±1.01[a]	0.44±0.04[a]	0.68±0.03[a]
t	27.164	18.146	15.811	8.763	20.731	14.832	20.582	22.274	28.991
P	0	0	0	0	0	0	0	0	0

TABLE 5: THE OVEREXPRESSION OF miR-9-5P REVERSED THE EFFECTS OF EVODIAMINE ON INFLAMMATION AND OXIDATIVE STRESS IN SK-N-SH CELLS INDUCED BY 6-OHDA (x̄±s, n=9)

Group	miR-9-5p	LD (HU/l)	GSH (mg/l)	IL-1β (pg/ml)	IL-6 (pg/ml)	IL-18 (pg/ml)
10 μmol/l EVO+miR-NC	1.00±0.05	18.04±1.37	78.44±4.94	10.08±0.75	14.24±0.76	13.75±0.85
10 μmol/l EVO+miR-9-5p	2.17±0.10[a]	40.02±4.01[a]	51.06±4.19[a]	17.21±1.38[a]	27.64±2.64[a]	32.82±3.40[a]
t	31.394	15.561	12.681	13.619	14.633	16.324
p	0	0	0	0	0	0

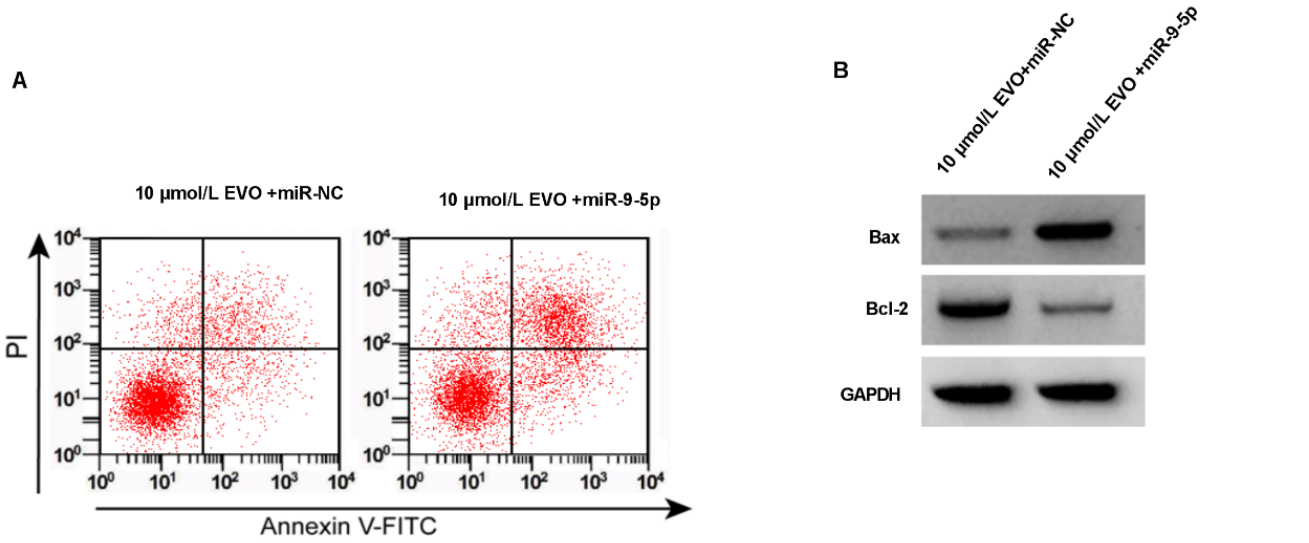

Fig. 3: MiR-9-5p overexpression attenuates evodiamine-induced inhibition on the apoptosis of 6-OHDA challenged SK-N-SH cells, (A): Analysis of cell apoptosis *via* flow cytometry and (B): Measurement of Bcl-2 and BAX proteins *via* Western blot

TABLE 6: miR-9-5P BOOST ABATED THE EFFECTS OF EVODIAMINE ON 6-OHDA EVOKED APOPTOSIS IN SK-N-SH CELLS

Group	Apoptosis rate (%)	BAX	Bcl-2
10 μmol/l EVO+miR-NC	10.53±0.92	0.32±0.04	0.80±0.04
10 μmol/l EVO+miR-9-5p	21.96±1.73[a]	0.60±0.05[a]	0.50±0.03[a]
t	17.5	13.119	18
p	0	0	0

The inflammatory reaction of neurons can aggravate the oxidative stress of cells, thereby inducing the apoptosis of neurons and promoting neuron injury. Research shows that HM can treat PD *via* multiple targets and multiple ways[17]. Additionally, research have shown that miRNA is abnormally expressed in the cell model of PD[18,19]. Whereas, whether Chinese HM can be used in combination with miRNA for PD therapy.

Previous study manifested that evodiamine can slow down the development of atherosclerosis by inhibiting infectious and inflammatory reaction[20]. Evodiamine alleviated renal ischemia-reperfusion injury in rats[21]. Evodiamine reduced the arthritis in rats by inhibiting inflammatory reaction[22]. However, the functions of evodiamine on PD remain unknown. Here, this work manifested that 6-OHDA exposure induce the activation of LDH but declined the content of GSH in neurons, suggesting that the cell model of PD was successfully induced. Functionally, we found that evodiamine treatment could dose-dependently arrest LDH activity and elevated GSH content in 6-OHDA-induced neurons, implying the potential inhibitory

function of evodiamine on oxidative stress in neurons during PD. In addition, the contents of inflammatory cytokines were declined by 6-OHDA in neurons, which were dose-dependently rescued by evodiamine treatment, suggesting that evodiamine was able to inhibit the inflammatory reaction in neurons. Moreover, we also discovered that 6-OHDA stimulation led to the increase of cell apoptosis, accompanied with the increased BAX and decreased Bcl-2 protein; moreover, evodiamine could protect neurons from 6-OHDA stimulated apoptosis. In all, evodiamine could inhibit 6-OHDA induced damage in the PD cell model.

MiR-9-5p is a functional miRNA. MiR-9-5p in podocytes induced by high glucose was increased and inhibition of its expression could promote podocyte proliferation and inhibit cell apoptosis induced by high glucose[23]. MiR-9-5p exhibited high level in hypoxic induced cardiomyocytes and could expedite oxidative and apoptotic injury of cardiomyocytes[24]. Inhibiting miR-9-5p suppressed neuron apoptosis induced glucose deprivation/reoxygenation[25]. Herein, we discovered an up-regulated miR-9-5p in 6-OHDA stimulated neurons, moreover, inhibition of miR-9-5p abated stimulated apoptotic, oxidative and inflammatory injury in neurons treated with 6-OHDA. Subsequently, it was found that evodiamine treatment resulted in miR-9-5p descend in 6-OHDA-stimulated neurons; moreover, its elevation abrogated the protective effects of evodiamine on 6-OHDA stimulated neurons, indicating that evodiamine could reduce the neuron damage *via* miR-9-5p.

In conclusion, evodiamine could inhibit 6-OHDA induced apoptotic, oxidative and inflammatory injury in the PD cell model *via* miR-9-5p, implying the potential application of evodiamine in PD therapy.

Conflict of interests:

The authors declared no conflict of interests.

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