Original Article

Protective effects of resveratrol liposomes on mitochondria in substantia nigra cells of parkinsonized rats

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Background: Parkinson's disease (PD) is a central nervous system degenerative disease. The progressive death of dopaminergic neurons is closely correlated to mitochondrial dysfunction. Resveratrol contains three hydroxyl groups, and has a strong neuroprotective effect. This study aimed to investigate the protective effect of resveratrol liposome on mitochondria of substantia nigra cells in Parkinsonized rats through experiment.

Methods: The investigators used 6-hydroxydopamine to establish the Parkinsonized rat model, and used resveratrol liposome from Polygonum cuspidatum (20 $mg \cdot kg^{-1}$) for gavage, up to a total volume of 1 mL, once-daily, for two weeks. After treatment, the levels of mitochondrial membrane potential, mitochondrial complexes I-IV, mitochondrial cytochrome C, apoptosis-inducing factor (AIF), PTEN-induced putative kinase 1 (PINK1), tumor necrosis factor-receptor-associated protein 1 (TRAP1) and phosphorylated TRAP1 in rat mesencephalic cells were detected according to the operation instructions of the kits.

Results: After two weeks of treatment, resveratrol liposomes could significantly enhance the activity of mitochondrial electron transfer chain complex I in the substantia nigra cells of Parkinsonized model rats, promote the expression of complex I subcomponent MT-ND1-37kD, improve mitochondrial membrane potential, inhibit the release of mitochondrial cytochrome C and apoptotic inducible factor, enhance the expression of mitochondrial functional protein PINK1, increase the phosphorylated TRAP1 level, and elevate the phosphorylated TRAP1/TRAP1 ratio.

Conclusions: Resveratrol liposome has positive effects on mitochondria in substantia nigra cells of Parkinsonized rats, and may be one of its pharmacological mechanisms.

Keywords: Parkinson's disease; resveratrol; liposomes; mitochondria; rat model

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Introduction

Parkinson's disease (PD) is a kind of central nervous system degenerative disease. Its main clinical manifestations are static tremor, bradykinesia and postural balance disorder. The typical pathological changes of PD include progressive and selective deaths of dopaminergic neurons in the substantia nigra of the midbrain, which induce a significant reduction of dopamine in the substantia nigra striatum pathways. The exact mechanism of selective death of dopaminergic neurons remains unknown, to date.

The progressive death of dopaminergic neurons is closely correlated to mitochondrial dysfunction. It has been found

that the activity of mitochondrial complex I in the substantia nigra of PD patients is generally lower by 30-40%, when compared to normal subjects, and this is accompanied by increased local oxidative stress in the substantia nigra (1,2). Experiment results have suggested that oxidative stress is related to the pathological process of PD (3,4). The coding products of nine familial PD pathogenic genes have been identified either as mitochondrial proteins themselves, or functionally correlated to mitochondria (5,6). Therefore, the protection of mitochondrial function, the prevention or reversal of mitochondrial dysfunction and the series of subsequent harmful reactions may provide more direct and effective treatment strategies and approaches for the clinical prevention and treatment of PD.

The investigators confirmed in a previous study that polygonum cuspidatum resveratrol liposome can significantly improve the behavioral abnormalities of 6-hydroxydopamine (6-OHDA)-induced Parkinsonized model rats, increase the number of cells, neurons and dopaminergic neurons in the substantia nigra, reduce cell apoptosis rates, improve the total antioxidant capacity of tissues, and reduce the total activity of reactive oxygen species (ROS) in tissues (7).

The general view is that resveratrol contains three hydroxyl groups, has a strong antioxidant activity as the basis of its pharmacological effects (8,9), and has a strong neuroprotective effect (10-14). It has been reported that resveratrol can repair the pathological damage of the blood-brain barrier (15,16). Meanwhile, some studies have confirmed that resveratrol can cross the blood-brain barrier, plays an antioxidant and anti-inflammatory role, and protects the blood-brain barrier and brain nerve of experimental animals (17,18). However, the medical dosage of resveratrol does not appear to be able to completely neutralize the oxygen free radical molecules formed in Parkinsonized model rats in the pathological state. Thus, it is speculated that there may be other mechanisms on the positive effects of resveratrol on mitochondria in the substantia nigra cells of Parkinsonized rats. In view of this, the present study determined whether resveratrol liposomes have direct effects on the mitochondrial function of substantia nigra cells in Parkinsonized model rats, in order to preliminarily reveal the targets of resveratrol in neuroprotection, and provide ideas for the clinical development of new drugs for treating neurodegenerative diseases, such as PD.

Methods

Extraction and identification of resveratrol from Polygonum cuspidatum and preparation and quality evaluation of resveratrol liposomes (7)

Polygonum cuspidatum decoction pieces (Hubei Pharmaceutical Company, China) were crushed, pretreated with cellulase enzymatic hydrolysis, roughly extracted by ethanol heating reflux method, separated and refined by AB-8 macroporous adsorption resin, purified by silica gel column and recrystallized by acetone. The acicular crystals were confirmed to be resveratrol monomer by melting point determination and high-performance liquid chromatography (HPLC) detection of the mixture with the control. The purity is over 99%. Soybean lecithin and cholesterol were accurately weighed in proper proportion (imported by Shanghai Dongshan Technology Co., Ltd., batch number 20060905, China). Resveratrol liposomes were prepared by thin film dispersion method. The encapsulation efficiency was 93.52%. TEM2000 transmission electron microscopy showed that most of the liposomes were spherical or elliptical, with particle sizes ranging 146–585 nm, and pH value of 6.87±0.13. The results of pharmacokinetic tests in mice and HPLC showed that the serum concentration of resveratrol liposome was higher than that of resveratrol at the same time, and the half-life of resveratrol liposome was longer than that of resveratrol (19,20).

Experimental animal grouping, model establishment method, model evaluation and drug processing

One hundred male Wistar rats, weighing 180–210 g, were provided by the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology (Lot number: TJLA-2006-043). These rats were randomly divided into five groups: normal group, sham operation group, model group, blank liposome group and resveratrol liposome group (n=20, each). The methods for model establishment and administration were based on a previous experiment (7). Experiments were performed under a project license (No. 2020060101) granted by Animal Use Committee of Beijing Institute of traditional Chinese medicine, in compliance with international, national or institutional guidelines for the care and use of animals.

Tissue acquisition

After deep anesthesia, the rats were quickly decapitated, and the brain was obtained and placed on an ice tray. Then, the midbrain was quickly separated and frozen in liquid nitrogen for subsequent testing.

UV spectrophotometric detection of mitochondrial complexes I-IV

The fresh substantia nigra tissues of rats were obtained, the mitochondria were extracted using the GenMed Animal Tissue Mitochondrial Isolation Kit, and the activity of the corresponding mitochondrial complexes was determined using the mitochondrial complex activity colorimetric method.

Western blot analysis of mitochondrial complex I content

Concentrated gum concentration was 5%, separation gum concentration was 12%, and protein sample size was 20 μg. After electrophoresis, the protein was transferred to PVDF membrane by electro-transfer method. Tris-Buffered Saline and Tween 20 (TBST) containing 5% skimmed milk powder (0.1% Tween-80, 100 mmol/L Tris, 0.9% NaCl pH 7.5) was used for overnight blocking. The first anti-Goat complex1 antibody (24-51 kD, 1:500, SANTA, SC-20493) was diluted with 0.2 μg/mL, incubated at room temperature for 2 hours, the second anti-rabbit anti goat IgG was diluted at a ratio of 1:1,000 and incubated at room temperature for 1 h.

Mitochondrial membrane potential detection by flow cytometry

A fluorescence probe JC-1 was used to detect the changes in mitochondrial membrane potential in the substantia nigra. When the mitochondrial membrane potential is relatively high, the JC-l would gather in the matrix of the mitochondria to form a polymer (J-aggregates), which produces red fluorescence. When the mitochondrial membrane potential is relatively low, the JC-1 does not accumulate in the matrix of mitochondria, and in this case, the JC-1 is in a monomer form, which produces green fluorescence. Based on these, changes in mitochondrial transmembrane potential in apoptotic cells were examined.

Western blot analysis of mitochondria and cytoplasmic cytochrome C

Mitochondria and cytoplasmic components were separated and extracted by differential centrifugation and density gradient centrifugation: nuclei, large membrane fragments and undissolved cells were precipitated after homogenate decomposition at 4 ℃ and 800 rpm for 5 min; 0.5 mL of Medium Buffer was added to another pre-cooled centrifugal tube, and 0.5 mL of supernatant of homogenate after centrifugation was taken carefully along the tube wall. The supernatant after centrifugation contained cytoplasmic components and was transferred to the new centrifugal tube. Mitochondria were deposited at the bottom of the tube. Mitochondrial precipitation was added with 0.2 mL of Wash Buffer heavy suspended mitochondrial precipitation, centrifuged at 4 ℃ for 10 min at 15,000 rpm, and supernatant was discarded.

Western blot was used to detect cytochrome C in mitochondria and cytoplasm respectively.

Detection of apoptosis-inducing factor (AIF), PTEN-induced putative kinase 1 (PINK1) and tumor necrosis factor-receptor-associated protein 1 (TRAP1) by Western blot

Concentrated gum concentration was 5%, separation gum concentration was 12%, and protein sample size was 20 μg. After electrophoresis, the protein was transferred to PVDF membrane by electro-transfer method. TBST containing 5% skimmed milk powder (0.1% Tween-80, 100 mmol/L Tris, 0.9% NaCl pH 7.5) was used for overnight blocking. The antibodies against the detected proteins were incubated at room temperature for 2 h, and the corresponding antibodies were incubated at room temperature for 1 h.

Detection of phosphorylated TRAP1 levels by immunoprecipitation and immunoblotting to reflect the ability of PINK1 to phosphorylate TRAP1

According to the operation instructions, tissue homogenate was lysed to obtain protein samples, and non-specific binding was removed by normal mouse IgG. TRAP1 protein was precipitated by Mouse HSP75/TRAP1 antibody (BD, 1:50) and Protein A+G Agarose (Santa Cruz). After washing, centrifugation and supernatant removal,

TRAP1 protein was added to 40 μL of x sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. The samples were centrifuged to the bottom of the tube by instantaneous high-speed centrifugation. The samples were treated in a boiling water bath for 5 minutes. SDS-PAGE was performed. The first antibody was Rabbit Phosphose antibody (CHEMICON, AB1603) detected by Western blot. The band was phosphorylated TRAP1.

Statistical methods

Data were analyzed using SPSS 11.0 statistics software. Inter-group comparison was conducted using one-way univariate analysis of variance (ANOVA). $P<0.05$ was considered statistically significant. All experiments were repeated three times.

Results

Effects of resveratrol liposomes on mitochondrial electron transfer chain complexes I-IV in substantia nigra cells of the midbrain in PD model rats

In the substantia nigra cells in PD model rats, mitochondrial respiratory chain complex I activity significantly decreased, and the reduction was more than half of that in the normal group and sham operation group ($P<0.01$), while the difference in activities of complexes II-IV among groups was not statistically significant ($P>0.05$). After two weeks of intervention with resveratrol liposomes, the mitochondrial respiratory chain complex I activity significantly enhanced in substantia nigra cells in PD model rats (*Figure 1A*).

The Western blot detection results of the three components of mitochondrial respiratory chain complex I [NADH dehydrogenase (ubiquinone) flavin protein 1, NADH dehydrogenase (ubiquinone) flavin protein 2, and NADH dehydrogenase subunit 1] were as follows: the levels of complex I-51kD and complex I-24kD encoded by nuclear genes were not significantly different among the groups, while the level of MT-ND1-37kD encoded by mitochondrial DNA significantly decreased in the model group. After two weeks of intervention with resveratrol liposome, the level of MT-ND1-37kD significantly increased in substantia nigra cells in PD model rats (*Figure 1B*,*C*).

Resveratrol liposome intervention significantly increased the percentage of cells with normal mitochondrial membrane potential in substantia nigra cells of PD rats

The percentage of cells with decreased mitochondrial membrane potential in substantia nigra cells was significantly higher in PD model rats, when compared to the normal group and sham operation group ($P<0.01$). After two weeks of intervention with resveratrol liposome, the percentage of cells with depolarized mitochondrial membrane potential significantly decreased in substantia nigra cells in PD model rats. That is, the percentage of cells with normal mitochondrial membrane potential significantly increased (*Figure 2*).

Resveratrol liposome significantly reduced the levels of cytochrome c and AIF in the cytoplasm of substantia nigra of PD rats

The levels of cytochrome C and AIF in the cytoplasm of substantia nigra cells in PD model rats significantly increased. However, after two weeks of intervention with resveratrol liposome, the levels of cytochrome C and AIF in the cytoplasm of mesencephalic substantia nigra cells significantly decreased (*Figure 3*).

Resveratrol liposome intervention normalizes the PINK1 level and the ratio of phosphorylated TRAP1/TRAP1 in substantia nigra of PD rats

The level of PINK1 in substantia nigra cells was significantly lower in PD model rats, when compared to rats in the normal group and sham operation group, while the level of TRAP1 was significantly higher, when compared to the normal group and sham operation group. Furthermore, the level of phosphorylated TRAP1 and the phosphorylated TRAP1/TRAP1 ratio significantly decreased. After two weeks of intervention with resveratrol liposomes, the level of PINK1 significantly increased in substantia nigra cells in PD model rats, the phosphorylated TRAP1 level significantly increased, but the phosphorylated TRAP1/TRAP1 ratio significantly decreased (*Figure 4*).

Discussion

The mechanism of chronic, progressive and selective deaths

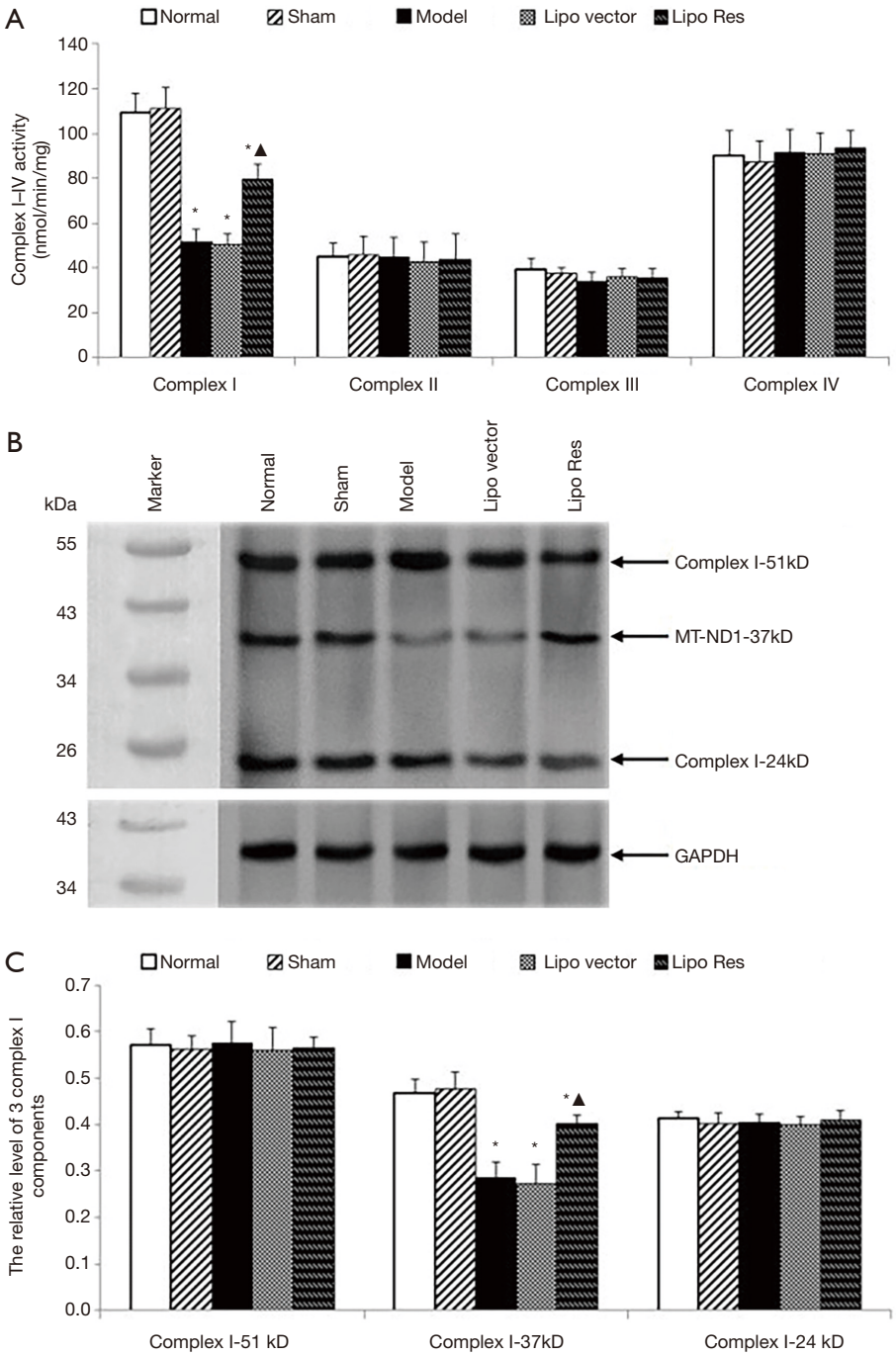

Figure 1 The activities of mitochondrial complexes I-IV and levels of the three subunits of complex I in substantia nigra cells in rats in the groups. (A) Mitochondria were extracted from the substantia nigra of rats in all groups, and the activities of complexes I-IV were determined by ultraviolet spectrophotometry. (B) Proteins were extracted from substantia nigra of rats in all groups, the relative contents of complex I-51kD, complex I-24kD, MT-ND1-37kD, which are the three subunits of complex I, were determined by Western blot, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. (C) The relative content of mitochondrial subunits complex I-51 kD, complex I-24 kD and MT-ND1-37kD in the substantia nigra in rats in the different groups. All result data were expressed as mean ± standard deviation (mean ± SD). *Compared with the normal group and sham operation group, $P<0.01$; ▲Compared with the model group and blank liposome group, $P<0.01$.

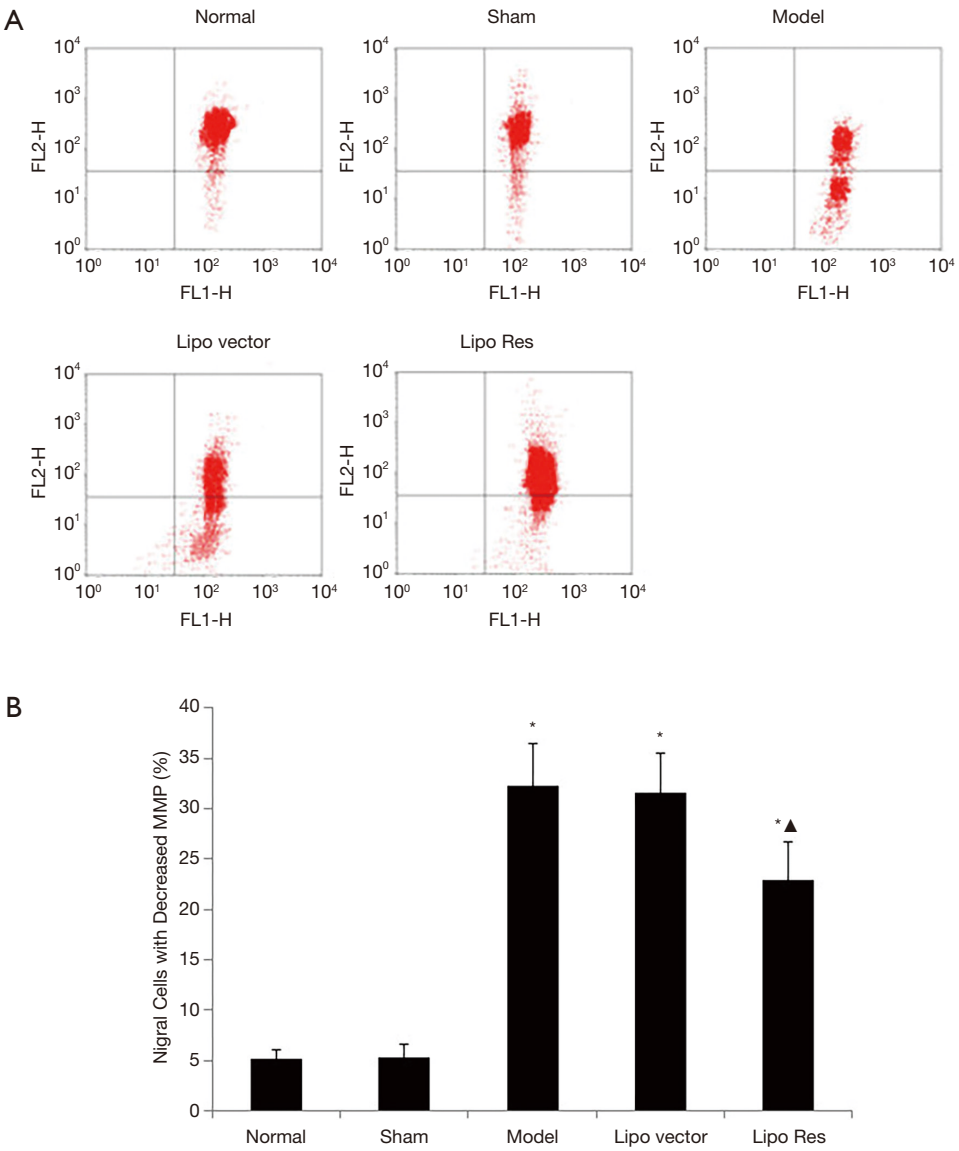

Figure 2 Detection results of mitochondrial membrane potential in substantia nigra cells in rats in different groups. (A) The mitochondrial membrane potential of substantia nigra cells in rats in all groups was measured by flow cytometry using the fluorescent probe JC-1. In the representative maps of all groups, the right upper quadrant reveals the percentage of cells with good mitochondrial membrane potential, while the right lower quadrant reveals the percentage of cells with changes (decrease) in mitochondrial membrane potential. (B) The percentage of cells with decreased mitochondrial membrane potential in substantia nigra cells in rats in all groups. All result data were expressed as mean ± standard deviation (mean ± SD). *Compared with the normal group and sham operation group, P<0.01; ▲Compared with the model group and blank liposome group, P<0.01.

of dopaminergic neurons in the substantia nigra in PD patients has been investigated. Increasing evidence suggests that apoptosis is the major mode of death of dopaminergic neurons, and its occurrence is correlated to the abnormal mitochondrial function of substantia nigra cells and the local hyperoxidative environment of the substantia nigra itself (21).

Some studies have confirmed that the mitochondrion was the integration center and executor of apoptotic signals, which initiates and completes the process of cell

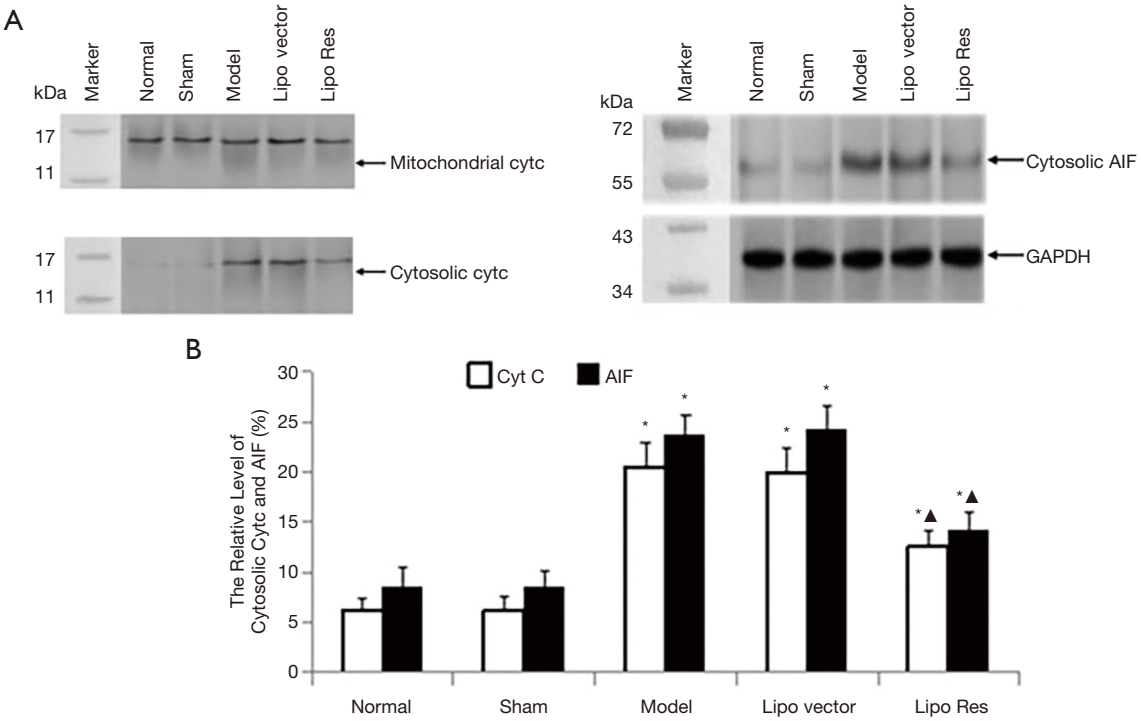

Figure 3 Contents of mitochondrial cytoplasmic cytochrome C (cytochrome C) and cytosolic cytochrome C and apoptosis-inducing factor (AIF) in substantia nigra cells in rats in the different groups. (A) The mitochondria and cytoplasm of substantia nigra cells were separated by differential centrifugation and density gradient centrifugation, and the relative contents of mitochondrial cytochrome C and AIF were detected by Western blot, with GAPDH as the internal control. (B) Relative content of cytosolic cytochrome C and AIF in substantia nigra cells in rats in the different groups. All result data were expressed as mean ± standard deviation (mean ± SD). *Compared with the normal group and sham operation group, P<0.01; ▲Compared with the model group and blank liposome group, P<0.01.

apoptosis, when various apoptotic factors accumulate to a certain extent (22). Therefore, the functional state of mitochondrion is an important factor in determining the fate of dopaminergic neurons in the substantia nigra in the pathogenesis of PD.

Mitochondrial respiratory chain complexes I-V are important for mitochondrial function, especially complexes I and III, which lead to the formation of superoxide. Some studies have proposed some evidences that resveratrol regulates respiratory chain complexes (23). A study revealed that a proper dose of resveratrol could inhibit the production of ROS in mitochondria by upregulating the active expression of mitochondrial respiratory chain complexes (24). Other researchers have revealed that resveratrol could upregulate the levels of mitochondrial complexes (I and V) in Ts65Dn neuronal progenitor cells (25), mitochondrial complexes (I, III, IV and V) in hippocampal cells in rat offspring (26), and mitochondrial complexes (I-V) in hippocampal cells in SAMP8 mice (14), thereby protecting mitochondria and slowing down the apoptosis. The results of the present study revealed that the activity of mitochondrial respiratory chain complex I significantly decreased in substantia nigra cells in PD rats induced by 6-OHDA. The contents of three functional proteins in the subcomponents of complex I were further investigated. The result revealed that the levels of complex I-51kD and complex I-24kD encoded by nuclear genes were not significantly different among the groups, while the level of MT-ND1-37kD encoded by mitochondrial DNA significantly decreased in the model group. Furthermore, the result revealed that in substantia nigra cells in PD model rats, not only the mitochondrial complex I activity, but also the expression of functional proteins synthesized by mitochondria decreased. In addition, the present study revealed that after two weeks of intervention with resveratrol liposome, the activity of mitochondrial respiratory chain complex I in substantia nigra cells significantly increased. This result is similar to other previous studies (14,23-26).

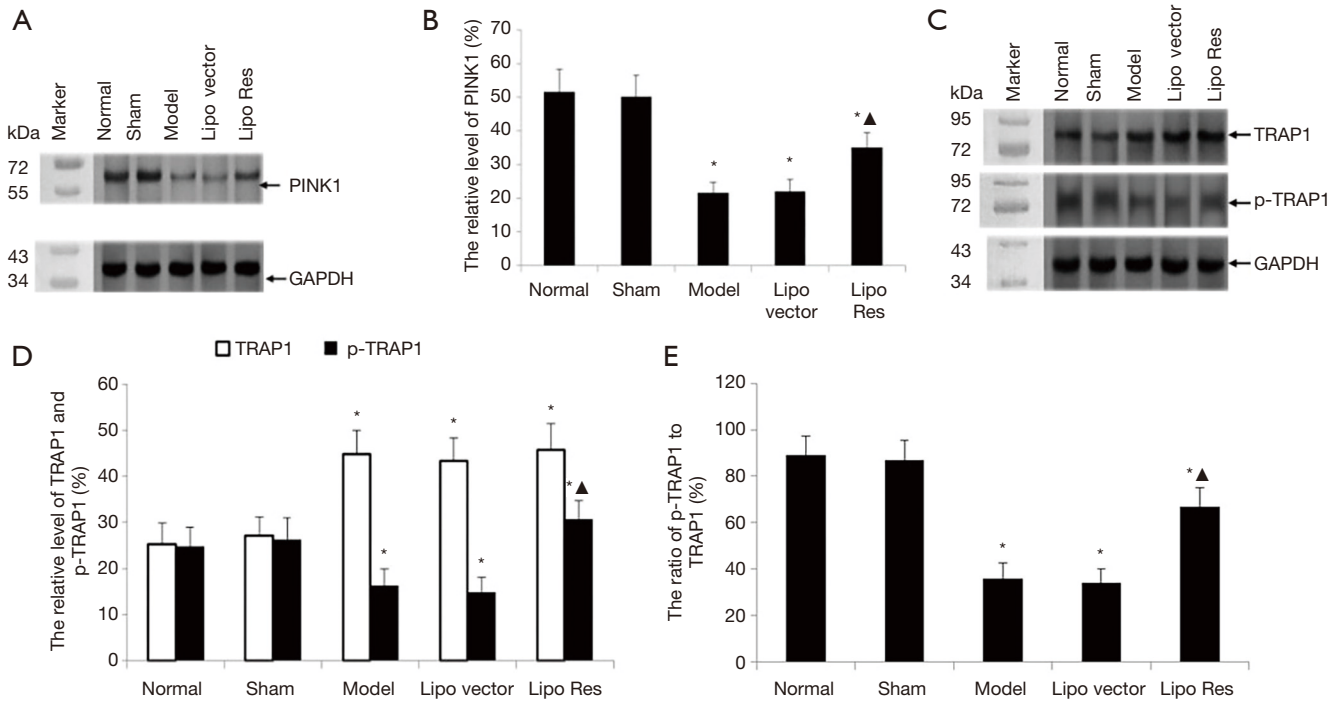

Figure 4 Contents of PTEN-induced putative kinase 1 (PINK1), tumor necrosis factor-receptor-associated protein 1 (TRAP1) and phosphorylated TRAP1 in substantia nigra cells in rats in different groups. (A) Content of PINK1 in substantia nigra cells in rats in different groups was detected by Western blot, with GAPDH as the internal control. (B) Relative content of PINK1 in substantia nigra cells in rats in different groups. (C) Contents of TRAP1 and phosphorylated TRAP1 (p-TRAP1) in substantia nigra cells in rats in different groups were detected by Western blot, with GAPDH as the internal control. (D) Relative content of TRAP1 and phosphorylated TRAP1 in substantia nigra cells in rats in the different groups. (E) The TRAP1/phosphorylated TRAP1 ratio in substantia nigra cells in rats in different groups was presented. All result data were expressed as mean ± standard deviation (mean ± SD). *Compared with the normal group and sham operation group, P<0.01; ▲Compared with the model group and blank liposome group, P<0.01.

Moreover, the level of MT-ND1-36kD, which is a complex I subunit, significantly increased. This result preliminarily reveals that resveratrol liposomes have protective effects on mitochondrial respiratory chain function in substantia nigra cells in Parkinsonized rats. Furthermore, this result also reveals that mitochondrial respiratory chain complex I activity and its subcomponent expression could be regulated by the uptake of drugs, which is one of the research targets of PD therapeutics.

When the mitochondrial transmembrane potential collapses, the mitochondrial membrane permeability increases, and lethal substances in the mitochondria, such as cytochrome C and AIF, are released into the cytoplasm, thereby causing apoptosis to be irreversible (27). In the present study, the percentage of cells with decreased mitochondrial membrane potential was significantly increased in substantia nigra cells in PD model rats. Correspondingly, the levels of cytochrome C, AIF and other proteins in the cytoplasm of substantia nigra cells in model rats also increased. After two weeks of intervention with resveratrol liposomes, the corresponding indicators significantly improved in these model rats. This suggests that resveratrol liposomes have certain pharmacological effects in maintaining the mitochondrial membrane potential of substantia nigra cells and preventing harmful downstream events in PD rats.

Previous studies revealed that PINK1 could maintain the normal mitochondrial membrane potential (28). TRAP1 has been proven to be closely correlated to the maintenance of normal mitochondrial function (29). Recent studies have revealed that TRAP1 may be the kinase substrate of PINK1, and that the maintenance effect of PINK1 on the mitochondrial membrane potential depends on the kinase activity of PINK1 to TRAP1 (28). The investigators

observed in the experiment that resveratrol liposomes could increase the levels of PINK1 and phosphorylated TRAP1 in substantia nigra cells in PD model rats, and elevate the phosphorylated TRAP1/TRAP1 ratio, which may be one of the molecular bases of the protective mitochondrial function of resveratrol liposomes in substantia nigra cells in Parkinsonized rats.

Oxidative stress is closely correlated to mitochondrial function. The main source of ROS is the mitochondrial respiratory chain. Mitochondrial respiratory chain dysfunction, especially the function defect of complex I, which is the "entrance enzyme" of the respiratory chain, prevents electrons from being transferred, resulting in the production of a large number of oxygen free radicals, and these oxygen free radicals in turn attack the mitochondrial membranes and respiratory chain enzyme molecules, forming a vicious circle. This can explain why the sole use of antioxidants, such as vitamin E, cannot prevent the progression of the disease (27). The previous study of the investigators revealed that resveratrol liposomes can decrease the total ROS activity in the substantia nigra of PD model rats, improve the total antioxidant capacity in tissues, improve the high oxidation state in the substantia nigra, increase the number of substantia nigra cells and dopaminergic neurons in the midbrain, and decrease the apoptotic rate (7). These results reveal that resveratrol liposomes have obvious antioxidant effects. The present study revealed that after two weeks of intervention with resveratrol liposomes, the activity of mitochondrial respiratory chain complex I in substantia nigra cells significantly increased. Therefore, it can be speculated that resveratrol liposomes resist the toxic effects of exotoxin 6-OHDA by protecting mitochondrial function and inhibiting the apoptosis of substantia nigra cells, thereby preserving the survival of dopaminergic neurons in the substantia nigra.

However, there are some limitations in our experiments. The present study is an experimental study of rats, and its experimental results may be biased from those in the human body. Furthermore, the sample size of the experimental rats was small. Hence, if a large sample study is conducted, there may be inconsistencies between the results of that study, when compared to the present study. Although many literatures have dealt with the research results of resveratrol in the treatment of encephalopathy, the permeability and drug concentration of resveratrol and resveratrol liposomes in the blood-brain barrier and the relationship between the therapeutic effect and blood drug concentration remain unknown. These problems would be discussed and investigated by our research team in the future.

Conclusions

In conclusion, resveratrol liposomes have effects on the mitochondria of substantia nigra cells, including improving its function, reducing oxidative stress injury, and accordingly promoting the survival of substantia nigra cells in PD model rats. Furthermore, resveratrol liposomes have active positive effects on the mitochondria of substantia nigra cells in Parkinsonized rats, which may be one of its pharmacological mechanisms.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/apm-19-426). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license (No. 2020060101) granted by Animal Use Committee of Beijing Institute of traditional Chinese medicine, in compliance with international, national or institutional guidelines for the care and use of animals.

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