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Research Paper

Leaf extract of *Rhus verniciflua* Stokes protects dopaminergic neuronal cells in a rotenone model of Parkinson's disease

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Abstract

Objectives The present study investigated the neuroprotective effects of *Rhus verniciflua* Stokes (RVS) leaf extract on rotenone-induced apoptosis in human dopaminergic cells, SH-SY5Y.

Methods Cells were pretreated with RVS extract for 1 h then treated with vehicle or rotenone for 24 h. Cell viability, cell cytotoxicity, cell morphology and nuclear morphology were examined by MTT assay, lactate dehydrogenase release assay, phase contrast microscopy and staining with Hoechast 33342, respectively. Reactive oxygen species were measured by 2′7′-dichlorofluorescein diacetate and fragmented DNA was observed by TUNEL assay. Mitochondrial membrane potential was determined by Rhodamine 123. Pro-apoptotic and anti-apoptotic proteins and tyrosine hydroxylase were analysed by Western blotting.

Key findings Results showed that RVS suppressed rotenone-induced reactive oxygen species generation, cellular injury and apoptotic cell death. RVS also prevented rotenone-mediated changes in Bax/Bcl-2 levels, mitochondrial membrane potential dissipation and Caspase 3 activation. Moreover, RVS pretreatment increased the tyrosine hydroxylase levels in SH-SY5Y cells.

Conclusions These findings demonstrate that RVS protects SH-SY5Y cells against rotenone-induced injury and suggest that RVS may have potential therapeutic value for neurodegenerative disease associated with oxidative stress.

Keywords apoptosis; dopaminergic cell; Parkinson's disease; *Rhus verniciflua*; rotenone

Introduction

Parkinson's disease (PD) is the second most common age-related progressive neurodegenerative disorder after Alzheimer's disease (AD). This devastating disease affects more than 1% of the population over 65 years old.[1] Because of its prevalence, it is expected to impose an ever-increasing social and economic burden on society. Pathologically, PD is characterized by the marked degeneration of dopaminergic neurons in the substantia nigra pars compacta. Although the etiology of PD is not completely understood, many pathogenic mechanisms, such as oxidative stress, mitochondrial dysfunction and apoptosis, have been shown to play important roles. Epidemiological studies have suggested that a variety of environmental factors, particularly pesticide exposure, increase the risk of PD.[2] Among pesticides, rotenone is a naturally occurring plant compound and a common insecticide used in vegetable gardens. It is also used to kill fish. Rotenone inhibits complex I and reproduces many features of PD, including selective dopaminergic degeneration, increased oxidative damage and α-synuclein aggregation.[2–4] The rotenone model reproduces most of the mechanisms that are thought to be relevant in PD pathogenesis and is thus suitable for testing of neuroprotective strategies.[4,5]

Nowadays, natural products with antioxidant capacities have received significant attention due to their diverse range of biological properties. The exploration of ethnopharmacologic treatments may be a cost-effective and promising for developing therapeutics for PD. *Rhus verniciflua* Stokes (RVS), a member of the Anacardiaceae family, is commonly known as the lacquer tree. It is used as a food additive and traditional herbal medicine for

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the treatment of cancer and stomach diseases.[6] RVS contains phenolic compounds such as gallic acid, protocatechuic acid, quercetin, fustin, fisetin, sulfuretin and butein, and has been reported to exert antioxidant,[7,8] anti-inflammatory,[9] anti-tumorigenic,[10] antiapoptotic[11] and anticancer effects.[12,13] Recently, we showed that detoxified stem bark extract of RVS regulates catecholamine biosynthesis and neurotrophic factors in rat brain and inhibits rotenone-induced apoptosis in SH-SY5Y cells.[14–16] Interestingly, the extracts from different parts of RVS, such as the root, fruit, heartwood and bark of the stem and trunk, have been reported to have a wide range of biological activities.[10,14,17–20]

The array of compounds in the plant is daunting, with wide-ranging chemical, physical and biological activities to match. The content of phytochemicals varies hugely among plant species. The concentrations of phytochemicals in different parts of the plant are not uniform, and different amounts may be present in the leaves, flowers, fruits, bark, heartwood and roots. Variations in the content of these compounds between plants of a given species and between seasons have also been found. These variations thus present numerous challenges to the utilization of plants but numerous opportunities for adding value to plant products. In Korea, the young leaves of RVS have been used as a folk remedy for health reinforcement.[21] However, the biological activities of the leaf extract of RVS are unknown to date. In this study, therefore, we attempted to elucidate for the first time the biological activity of RVS leaf extract by investigating its effect on cell injury in rotenone-treated human dopaminergic cells, SH-SY5Y, and evaluating whether RVS may be of potential therapeutic value in the treatment of neurodegenerative diseases, such as PD.

Materials and Methods

Materials

Dulbecco's Modified Eagle Medium: nutrient mix F-12 (1 : 1, DMEM/F-12), foetal bovine serum (FBS), penicillin and streptomycin were obtained from Gibco BRL (Gaithersburg MD, USA). 2′7′-dichlorofluorescein diacetate (DCF-DA), DMSO, Hoechst 33324, 4,6-diamidino–2-phenylindole (DAPI) and Rhodamine 123 were obtained from Sigma-Aldrich (St Louis, MO, USA). Anti-tyrosine hydroxylase (TH) antibody was purchased from Affinity BioReagents, Inc. (Golden, CO, USA). Caspase 3, Bcl-2 and Bax antibodies were obtained from Santa Cruz (Santa Cruz, CA, USA). Anti-actin antibody was purchased from Biomeda Crop (Foster City, CA, USA). WEST-ZOL plus was obtained from Intron Biotech (Seongnam, Korea). Bicinchoninic acid (BCA) protein assay kit was obtained from Pierce (Rockford, IL, USA). Protein inhibitor cocktail was obtained from Calbiochem (Darmstadt, Germany). Cytotoxicity detection kit (LDH assay) was purchased from Roche Applied Science (Rotkreuz, Switzerland). DeadEnd™ fluorometric TUNEL system was purchased from Promega Corporation (Madison, WI, USA).

Preparation of RVS leaf extract

Fresh leaves of RVS were collected in Cheogwon, Chonbuk, Republic of Korea in the month of April and authenticated by Professor Myung-Kon Kim, Department of Bio-food Technology, Chonbuk National University, South Korea. A voucher specimen was deposited at Chonbuk National University. The leaves were dried in an oven at 60°C for 3 days and ground into fine powder. A quantity of 100 g of the powdered leaves was extracted with 1 l of methanol (70%) in a ratio of 1 : 10 w/v for 1 week at room temperature. The extract was filtered through Whatman No.1 filter paper and concentrated using a rotary vacuum evaporator. The concentrate was freeze dried and its yield was 4.8%. The final extract was a dark green powder. This powder was then dissolved in PBS and filtered through 0.2 µm membrane filter (Millipore, Bedford, MA, USA) and stored at 4°C.

Analysis of leaf extract

RVS leaf extract was analysed by HPLC using a Sycam HPLC system (Sycam, Gilching, Germany) equipped with a S2100 pump, S5200 autosampler and S3210 UV photodiode array detector. The column used in this study was YMC-Pack Pro C_{18} RS (250 mm × 4.6 mm i.d. S-5 µm, 8 nm, YMC Co. Ltd, Kyoto, Japan). A linear solvent gradient of the binary mobile phase (solvent A, 0.1% formic acid in HPLC grade water; solvent B, 0.1% formic in HPLC grade acetonitrile) during HPLC analysis was applied as follows (total 60 min): 92% A/8% B at 0 min, 89% A/11% B at 4 min, 76% A/24% B at 25 min, 72.5% A/27.5% B at 27 min, 40% A/60% B at 50 min, 92% A/8% at 52 min and 92% A/8% at 60 min. The flow rate was 1.0 ml/min and the injection volume was 20 µl. The detector was set at 230 nm. Peaks were identified by co-elution with authentic standards.

Cell culture and treatments

The human dopaminergic neuronal cell line, SH-SY5Y, was obtained from ATCC (Rockville MD). Cells were cultured in DMEM/F12 medium (Gibco, Gaithersburg) supplemented with 10% FBS and penicillin (100 units/ml)–streptomycin (100 µg/ml) at 37°C in 5% CO_2. Media were changed every 2 days. To examine possible toxic effects, SH-SY5Y cells were treated with the RVS leaf extract in a concentration ranging from 0.1 to 100 µg/ml for 24 h. Similarly, cells were treated with rotenone at concentrations ranging from 0.5 to 20 µM for 24 h. Samples of 1 and 10 µg/ml of RVS extract, which was non-toxic, and 5 µM rotenone were chosen to evaluate the neuroprotective effects by examining cell viability. RVS extract was added 1 h prior to treatment with rotenone. In a single experiment each treatment was performed in triplicate.

Analysis of cell viability

Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. SH-SY5Y cells were seeded in 96-well plates at a density of 1×10^4 cell/well and incubated for 24 h prior to experimental treatments. The cells were then subjected to the treatments of interest. After 24 h incubation, MTT (0.5 mg/ml) was added to each well. Following an additional 3 h incubation at 37°C, 100 µl of DMSO was added to dissolve the formazan crystals. The absorbance was then measured at 540 nm using a VERSAmax microplate reader (Molecular Devices, CA, USA). Wells without cells were used as blanks and were subtracted as background from each sample. Results were expressed as a percentage of control.

Lactate dehydrogenase release assay

Cells dying by apoptosis or necrosis release lactate dehydrogenase (LDH) into the supernatant. The amount of LDH in the supernatant was measured with a cytotoxicity detection kit (Roche). Briefly, the cells (1×10^4 cell/well) were seeded in 96-well plates and then treated with rotenone for indicated periods after pre-treatment with or without RVS extract for 1 h. For analysis, 100 µl of the supernatant was extracted from each well and placed in separate wells of a new 96-well plate. Then 100 µl of catalyst solution was added to each well and the plate was incubated at 37°C for 30 min. Absorbance was measured at 490 nm using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA). Total cellular LDH was determined by lysing the cells with 2% Triton X-100 (high control). The assay medium served as a low control and was subtracted from all absorbance measurements. The cytotoxity was calculated according to the following equation:

$$\text{cytotoxicity } (\%) = (\text{exp.value} - \text{low control}) / (\text{high control} - \text{low control}) \times 100$$

Measurement of intracellular reactive oxygen species

Production of ROS was measured using an oxidation-sensitive fluorescent probe 2′7′-dichlorofluorescein diacetate (DCF-DA, Sigma-Aldrich, St Louis, MO) method, which is based on the ROS-dependent oxidation of DCF-DA to the highly fluorescent compound dichlorofluorescein (DCF). The cells (1×10^4 cell/well) were cultured in 96-well plates and then treated with 5 µM rotenone for 24 h after pre-treatment with or without RVS extract for 1 h. Medium was removed and cells were washed twice with PBS. After washing, the cells were incubated with DCF-DA (10 µM) for 30 min at 37°C in the dark. Cellular fluorescence was measured in a fluorescence microplate reader (Spectra Max Gemini EM, Molecular Devices, Sunnyvale, CA, USA) at excitation wavelength 485 nm and emission wavelength 535 nm.

Nuclear staining with Hoechst 33342

Nuclear morphology was assessed by staining with the supravital DNA dye, Hoechst 33342. The cells (1×10^3 cells/well) were seeded in an eight-well chamber slide for 24 h and then treated with rotenone for indicated periods after pre-treatment with or without RVS extract for 1 h. The cells were washed twice with PBS and then fixed in 4% paraformaldehyde for 15 min. After two rinses with PBS, the cells were stained with 10 µg/ml DNA dye Hoechst 33342 in PBS for 30 min at 37°C. Slides were washed twice with PBS and examined under a fluorescence microscope (Nikon, Eclipse TE 2000-U, Japan) and photographed at 100 × magnification.

TUNEL assay

For in-situ detection of fragmented DNA, the TUNEL assay was performed using a DeadEnd™ Fluorometric TUNEL System (Promega Corporation, USA). Cells were cultured on an eight-well chamber slide at a density of 1×10^3 cells/chamber. After treatment with rotenone and RVS extract as indicated, cells were washed with PBS and fixed by incubation in 4% paraformaldehyde for 20 min at 4°C. The fixed cells were then washed and permeabilized with 0.2% Triton X-100 in PBS for 5 min. After rinsing with PBS, the cells were incubated with terminal deoxynucleotidyl transferase recombinant (rTdT) enzyme-catalysed reaction and nucleotide mixture for 60 min at 37°C in the dark and then immersed in stop/wash buffer for 15 min at room temperature. The cells were then washed with PBS to remove unincorporated fluorescein-12-dUTP. After washing, the nuclei were counterstained with 1 µg/ml DAPI solution for 15 min in the dark. The cells were observed with a fluorescence microscope (Nikon, Eclipse TE 2000-U, Japan) and photographed at 100 × magnification.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was determined using the fluorescent dye Rhodamine 123. Briefly, the cells were treated with rotenone for indicated periods after pre-treatment with or without RVS extract for 1 h. Cells were washed with PBS and incubated with 10 µg/ml Rhodamine 123 for 60 min at 37°C. The cells were washed and monitored by fluorescence microscope (Nikon, Eclipse TE 2000-U, Japan) and photographed. The fluorescence intensity was determined using a Spectra Max Gemini EM fluorometer (Molecular Devices, Sunnyvale, CA, USA) at 490 nm excitation and 515 nm emission.

Immunoblotting

After treatment, cells were washed once with PBS and then lysed using ice-cold RIPA buffer with protease inhibitor cocktail. Cell lysates were centrifuged at 12 000 rpm for 25 min and the protein concentrations were determined by the BCA method using BSA as standard. The proteins were separated by 10% SDS-PAGE and transferred to a polyvinylidine difluoride membrane. The membrane was blocked with 5% (v/v) non-fat dry milk in tris-buffered saline with Tween 20 (TBS-T) (10 mM Tris–HCl, 150 mM NaCl and 0.1% Tween 20, pH 7.5) and incubated with primary antibody for Bcl-2, Bax (1 : 1000 dilution), Caspase-3, TH (1 : 1000 dilution), and Actin (1 : 4000 dilution) for overnight at 4°C. The membrane was washed in TBS-T and incubated for 2 h at room temperature with horseradish peroxidase-conjugated secondary antibody. To reveal the reaction bands the membrane was reacted with a WEST-ZOL (plus) Western blot detection system (Intron Biotechnology, Inc., Korea) and exposed on X-ray film (BioMax MS-1, Eastman Kodak, USA).

Statistical analysis

The data were expressed as the means ± SD. Statistical significance was assessed with one-way analysis of variance followed by a post-hoc (Bonferroni) test for multiple group comparisons. Differences with P values less than 0.05 were considered statistically significant.

Result

Effects of RVS and rotenone on the survival of SH-SY5Y cells

We first examined whether RVS alone or rotenone alone were toxic to the human dopaminergic cell line, SH-SY5Y. Cells were treated with various concentrations of RVS (0.1, 0.5, 1, 10, 50 and 100 μg/ml) and rotenone (0.5, 1, 5, 10 and 20 μM) for 24 h and cell survival was determined by MTT assay. As shown in Figure 1a, when exposed to RVS concentrations of 10 μg/ml or lower, the viability of SH-SY5Y cells was the same as untreated control cells. However, a decrease of cell viability was observed with 50 and 100 μg/ml RVS extract. As shown in Figure 1b, rotenone

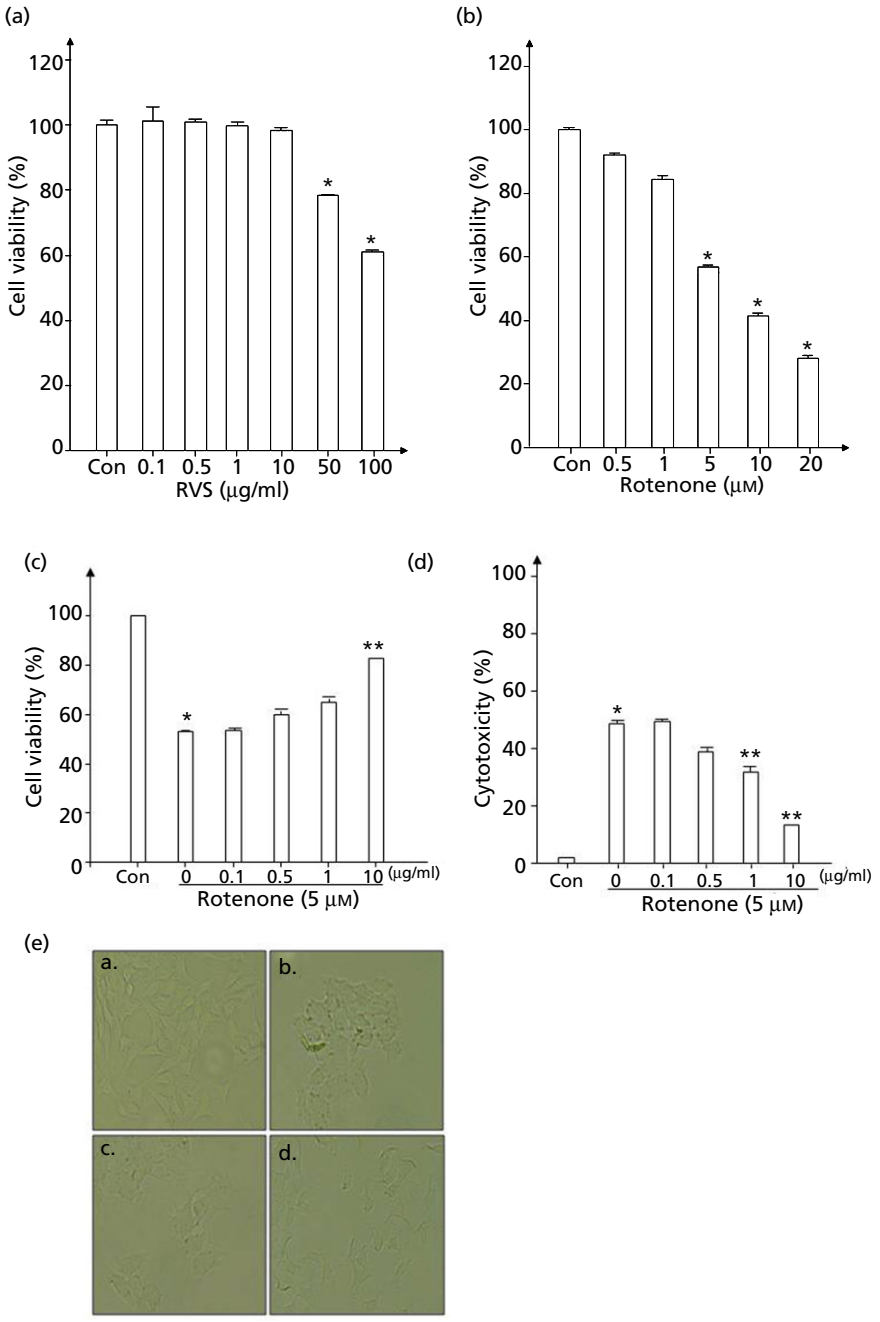

Figure 1 Effect of (a) RVS leaf extract or (b) rotenone on cell viability. (c) Protective effect of RVS leaf extract on rotenone-induced cytotoxicity in SH-SY5Y cells. Cell viability was assessed by the MTT assay. (d) Cells were pre-treated with different concentration of RVS leaf extract for 1 h then treated with 5 μM of rotenone for 24 h. Cell toxicity was measured by LDH assay. (e) Effects of RVS leaf extract on rotenone-induced morphological alterations in SH-SY5Y cells. Morphological studies were conducted by phase-contrast microscopy. (a) Control cells without any treatment, (b) 5 μM rotenone, (c) cells pretreated with 1 μg/ml RVS leaf extract for 1 h then treated with rotenone (5 μM). The data are expressed as mean ± SD of three experiments. Con, untreated control. *$P < 0.05$ versus control (Con) group. **$P < 0.05$, compared to rotenone-treated group.

induced dose-dependent cytotoxicity in SH-SY5Y cells. In the presence of 5 μM rotenone, there was only 56.68% of viable cells as compared to control cells. Consequently, cytotoxic induction with 5 μM rotenone for 24 h was used in the subsequent experiments.

RVS protects against rotenone-induced cytotoxicity

Next, we attempted to determine the effects of RVS on neuronal protection using the MTT reduction assay. To determine the protective effects of RVS against rotenone-induced loss of cell viability, SH-SY5Y cells were pre-treated with 0.1, 0.5, 1 and 10 μg/ml RVS extract for 1 h, followed by 5 μM rotenone for 24 h. As shown in Figure 1c, in the cells pre-treated with RVS (1 and 10 μg/ml) the survival rate was increased from 54% (rotenone-treated group) to 65.12 and 82.69%, respectively, whereas RVS (0.1 and 0.5 μg/ml) did not exert any protective effect. Rotenone-induced loss of cell viability was significantly attenuated by RVS treatment.

To further investigate the protective effect of RVS, the release of LDH was measured (Figure 1d). LDH release is increased as the number of dead cells increases. As shown in Figure 1d, release of LDH was increased significantly after exposure to 5 μM rotenone, indicating that rotenone caused cytotoxicity in SH-SY5Y cells. In contrast, RVS-treated cells (1, 10 μg/ml) showed decreased release of LDH compared with the rotenone-exposed cell group. The protective effect of RVS on rotenone-induced cytotoxicity determined by LDH assay was similar to that determined by MTT assay. RVS maintained the viability of cells against the neurotoxicity induced by rotenone, suggesting a protective effect of RVS.

The effects of RVS were also confirmed by morphological observation (Figure 1e). Morphological changes were observed in SH-SY5Y cells when treated with 5 μM rotenone for 24 h. The surviving cells showed a reduction of cytoplasm and diminution of tack on the plate, in contrast to the normal morphology of cells in the untreated control group. This effect was remarkably reversed when the cells were exposed to rotenone in the presence of RVS (1 and 10 μg/ml), indicating that RVS offered protection to the rotenone-induced damage in SH-SY5Y cells.

RVS ameliorated rotenone-induced oxidative stress

To determine the changes in intracellular ROS in human dopaminergic cells during rotenone-induced cell death and RVS-mediated protection, we measured ROS production in SH-SY5Y cells using fluorescent dye DCF-DA. As shown in Figure 2, the levels of intracellular ROS markedly increased (2.5 fold) after treatment with rotenone. However, pretreatment with RVS (1, 10 μg/ml) significantly decreased rotenone-induced ROS production 2.0- and 1.4–fold, respectively.

Effects of RVS against rotenone-induced apoptosis

The protective effects of RVS against rotenone-induced apoptosis were examined by Hoechst 33342 staining and TUNEL assay. Nuclear staining with Hoechst 33342

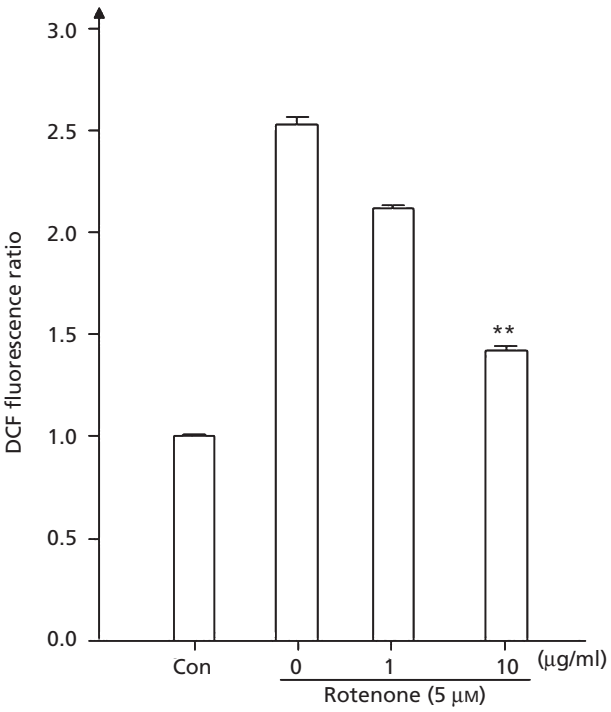

Figure 2 Effects of RVS leaf extract on rotenone-induced ROS generation in SH-SY5Y cells. Cells were pretreated with RVS leaf extract (1, 10 μg/ml, 1 h) then treated with vehicle or rotenone (5 μM, 24 h). Intracellular ROS accumulation was measured using the fluorescence probe DCF-DA. The fluorescence intensity was determined using a fluorescence microplate reader at 485 nm excitation and 535 nm emission. Values are presented as mean ± SD of three experiments. Con, untreated control. $*P < 0.05$ versus rotenone-treated group.

demonstrated that control SH-SY5Y cells had regular and round-shaped nuclei (Figure 3a). However, rotenone-treated cells (Figure 3b) exhibited highly condensed and fragmented nuclear morphologies, characteristics of apoptosis. In contrast, RVS pre-treatment inhibited these apoptotic features (Figure 3c and d).

Rotenone was shown to induce apoptosis by causing DNA strand breaks, as determined by TUNEL assay (Figure 4b). Pretreatment of RVS significantly inhibited rotenone-induced apoptosis (Figure 4c and d). These events were further confirmed by counterstaining with DAPI (Figure 4e–h). These results demonstrate that RVS decreased levels of cell death, nuclear condensation and DNA fragmentation, and indicate that RVS has an anti-apoptotic effect in SH-SY5Y cells.

RVS prevents rotenone-induced reduction of the mitochondrial membrane potential

The diffusion and accumulation of Rhodamine 123 in mitochondria is proportional to the degree of mitochondrial membrane potential (MMP).[22–24] A collapse of the mitochondrial *trans*-membrane potential has been linked to several models of apoptosis.[25] Examination of whether apoptosis in rotenone-treated cells and its prevention by RVS involves MMP changes was carried out using Rhodamine 123. The changes in fluorescent intensity of Rhodamine 123 were

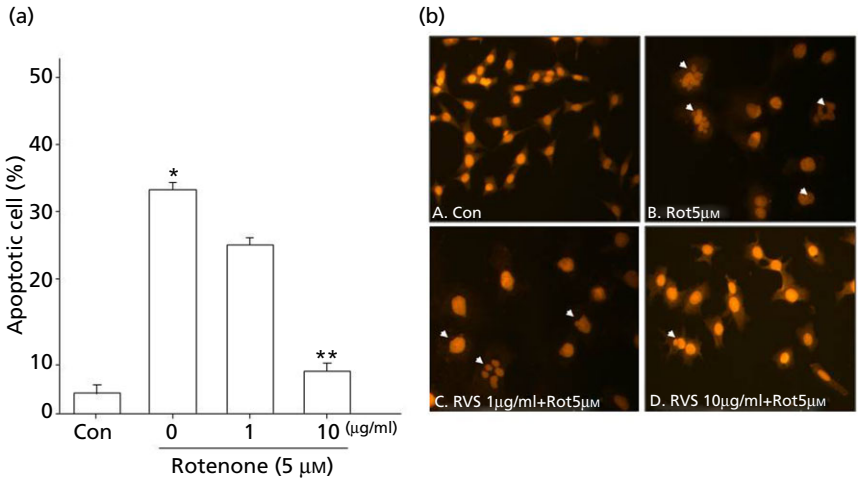

Figure 3 Effect of RVS leaf extract on the rotenone-induced changes in apoptotic nuclear morphology in SH-SY5Y cells. Apoptotic nuclear morphology was detected by Hoechst 33342 staining in (a) cells treated with vehicle, (b) 5 μM rotenone-treated cells, (c) cells treated with 1 μg/ml RVS leaf extract followed by 5 μM rotenone and (d) cells treated with 10 μg/ml RVS leaf extract followed by 5 μM rotenone. Arrows identify cells with condensed or fragmented nuclei, characteristic of apoptosis. Pictures were taken using a fluorescent microscope and photographed at 100 × magnification. (e) Quantification of apoptosis based on nuclear condensation or fragmentation. Data are expressed as mean ± SD of three experiments. Con, untreated control. *$P < 0.05$ versus control group. **$P < 0.05$, compared to rotenone-treated group.

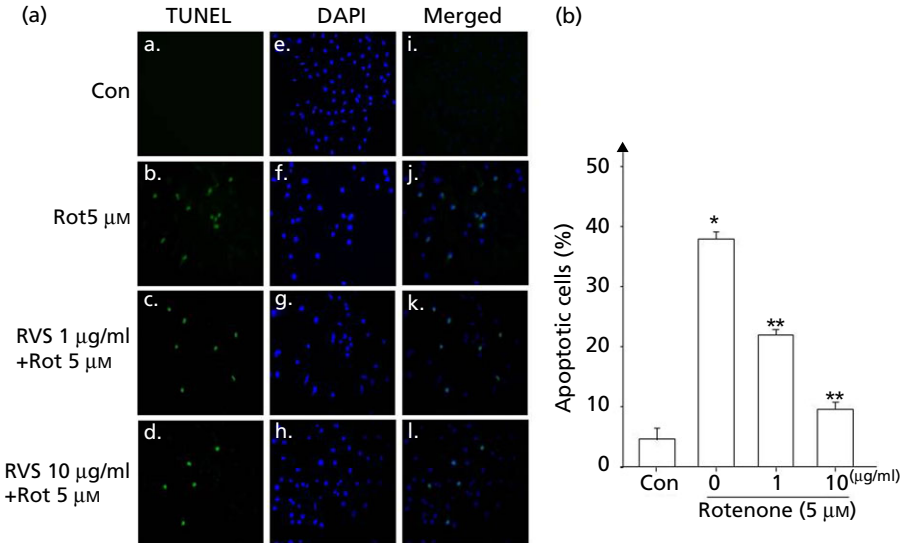

Figure 4 Effect of RVS leaf extract on rotenone-induced cell apoptosis in SH-SY5Y cells. Cell apoptosis was detected by staining with TUNEL (a–d), DAPI (e–h) and merged (i–l).Cells were pre-treated with leaf extract of RVS (1, 10 μg/ml, 1 h) then treated with rotenone (5 μM, 24 h). Each image is representative of three experiments. Pictures were taken using a fluorescent microscope and photographed at 100 × magnification. (e) Quantification of the number of TUNEL-positive cells as percentage of DAPI-positive cells. Data are expressed as mean ± SD of three experiments. Con, untreated control. *$P < 0.05$ versus control group. **$P < 0.05$, compared to rotenone-treated group.

measured and are presented in Figure 5a. As shown in Figure 5, when SH-SY5Y cells were exposed to 5 μM rotenone for 24 h, the mitochondrial membrane potential was significantly decreased to 45.23% (Figure 5a). However, the cells incubated with RVS (1, 10 μg/ml) prior to the addition of rotenone showed a marked increase in the MMP, by 59.04 and 68.18%, respectively, as compared with rotenone-treated cells (Figure 5). These results show that RVS extract suppresses the rotenone-induced decrease of MMP.

Effects of RVS on Bcl-2 and Bax protein expression in rotenone-treated SH-SY5Y cells

We investigated whether pro-apoptotic and anti-apoptotic gene expression were affected by RVS. Pro-apoptotic gene Bax and anti-apoptotic gene Bcl-2 protein levels were measured by Western blot. As illustrated in Figure 6a, rotenone enhanced the expression of Bax and concomitantly decreased the levels of Bcl-2. This resulted in a high Bax-to-Bcl-2 ratio,

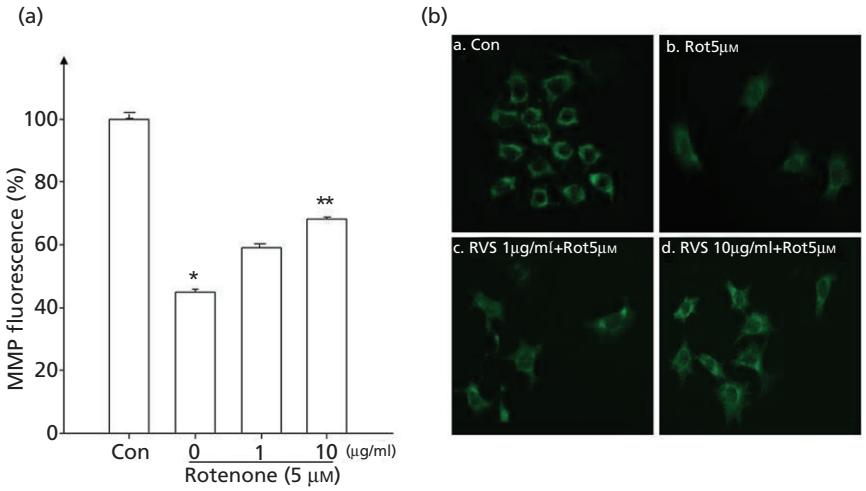

Figure 5 Effect of RVS leaf extract on rotenone-induced decrease of mitochondrial membrane potential. SH-SY5Y cells were pretreated with RVS leaf extract (1, 10 μg/ml) for 1 h followed by 5 μM rotenone for 24 h. Cells were incubated with Rhodamine 123 and the fluorescence intensity was determined using a fluorescence microplate reader at 490 nm excitation and 515 nm emission (a), membrane potential was monitored by fluorescent microscope (b). The reduced fluorescence of Rhodamine 123 was determined as the reduced mitochondrial membrane potential. Results are expressed as mean ± SD of three independent experiments. *$P < 0.05$ versus control group. **$P < 0.05$, compared to rotenone-treated group.

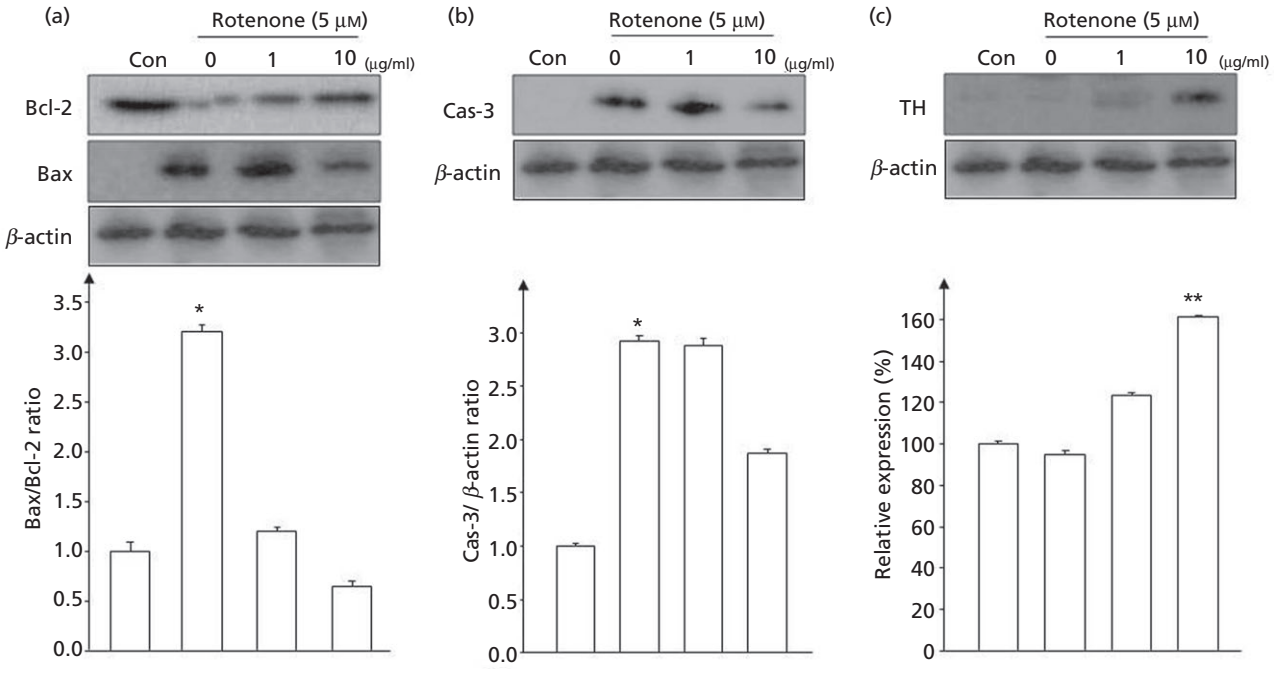

Figure 6 RVS leaf extract prevents rotenone-induced changes in the expression levels of apoptotic proteins and TH. Effects of RVS leaf extract on (a) Bcl-2 and Bax expression, (b) Caspase-3 expression and (c) TH expression. SH-SY5Y cells were pretreated with RVS (1, 10 μg/ml) for 1 h followed by 5 μM rotenone for 24 h. Expression of Bcl-2,Bax, Caspase-3 and TH was assessed by immunoblots and the intensity of each band was estimated by densitometric analysis. Actin was used as an internal loading control. Results are expressed as mean ± SD of three independent experiments. *$P < 0.05$ versus control group. **$P < 0.05$, compared to rotenone-treated group.

indicating that the rotenone-induced apoptosis in SH-SY5Y cells is probably mediated by the mitochondrial pathway. This effect was markedly attenuated by RVS. RVS pretreatment substantially reversed the changes of these ratios, especially the ratio of Bax to Bcl-2.

RVS decreases rotenone-induced Caspase 3 activation

We further investigated the effect of RVS on Caspase signalling. Activation of Caspase 3 has been implicated in

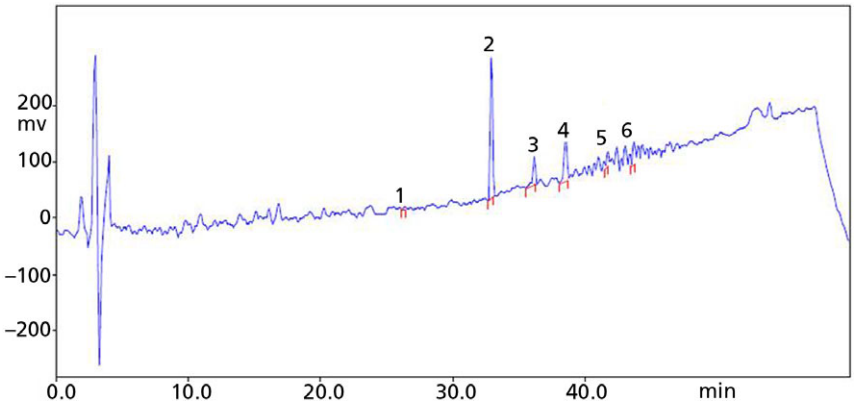

Figure 7 HPLC chromatogram of RVS leaf extract. Peaks: (1) fustin, (2) unknown, (3) fisetin, (4) sulfuretin, (5) quercetin, (6) butein.

Table 1 Concentration of flavonoids in RVS leaf extract

Flavonoids	Concentration (mg/100 g of extract)
Fustin	34.73 ± 0.05
Fisetin	470.72 ± 0.68
Sulfuretin	739.86 ± 0.97
Quercetin	192.00 ± 0.34
Butein	392.18 ± 0.71

The data are shown as mean ± SD of three replications.

dopaminergic apoptotic cell death. We evaluated whether Caspase 3 expression was affected by RVS. As shown in Figure 6b, rotenone treatment increased Caspase 3 activity 2.0-fold, but it was dramatically inhibited by RVS (10 μg/ml). Our data suggest that rotenone activates Caspase 3 in SH-SY5Y cells but that this activation is suppressed by RVS.

RVS modulates TH expression in rotenone-treated SH-SY5Y cells

As TH plays a key role as a rate-limiting enzyme in the dopamine biosynthesis pathway, we investigated the effect of RVS on protein level expression of this enzyme in SH-SY5Y cells. As shown in Figure 6c, rotenone treatment reduced TH expression. However, RVS pretreatment increased TH protein levels compared with the rotenone-treated group.

Identification of active compounds contained in RVS leaf extract

Figure 7 illustrates the HPLC chromatogram of the RVS leaf extract. Among the peaks observed in the chromatogram, five peaks have been identified as fustin (1), fisetin (3), sulfuretin (4), quercetin (5) and butein (6). The concentrations of these flavonoids in the tested extract are summarized in Table 1.

Discussion

Over the past decade, accumulating evidence has suggested an important role for environmental factors such as exposure to pesticides in the pathogenesis of some neurodegenerative diseases. Among these pesticides, rotenone has been constantly reported as being associated with an increased risk of PD. Indeed, a common denominator in the pathogenesis of PD by rotenone is the involvement of oxidative stress-mediated apoptotic processes.[2,26] Many studies have shown that oxidative stress is an important mediator of cellular damage in a various neurological disorders, including PD. Thus, inhibition of oxidative injury and subsequent dopaminergic neuronal loss may offer prospective therapeutic benefits for PD and other oxidative stress-related neurodegenerative disorders.

In the present study, we showed a protective effect of RVS leaf extract against rotenone-induced cell death in the human dopaminergic cell line, SH-SY5Y. This effect may be attributed, at least in part, to the presence of flavonoids in RVS leaf extract. HPLC analysis showed the presence of several flavonoids in the extract, such as fustin, fisetin, sulfuretin, quercetin and butein. These flavonoids have various pharmacological actions.[27,28] Interestingly, quantitative data show that RVS leaf extract contains high concentrations of sulfuretin. Recently, Lee *et al.*[28] reported that sulfuretin is one of the major flavonoids isolated from RVS, and this compound possesses strong antioxidant and anti-inflammatory effects. In addition, our results demonstrate that RVS exerts its neuroprotective effect through ROS scavenging (Figure 2). These results indicate that the antioxidative effect is one of the possible mechanisms for protection against ROS-induced cell damage. The extract of RVS decreased rotenone-induced cell death in SH-SY5Y cells. Apoptosis is the process of cell death, and is characterized by cell shrinkage, nuclear condensation, DNA fragmentation and membrane blabbing. These apoptotic features were detected in situ by Hoechst 33342, TUNEL and DAPI staining (Figures 3 and 4). Interestingly, RVS (10 μg/ml) significantly attenuated these features, indicating that RVS may possess an inhibitory effect on rotenone-induced apoptosis.

Neuronal death by apoptosis is a key contributing factor in neurodegenerative disorders such as PD. Apoptosis is initiated by a variety of external stimuli and molecular events such as oxidative stress, mitochondrial permeability transition, release of cytochrome c and activation of caspases. The Bcl-2 family consists of pro-apoptotic (for example Bax) and anti-apoptotic (for example Bcl-2) members. While the exact mechanisms remain elusive, it has been suggested that the

balance of pro- and anti-apoptotic Bcl-2 proteins decides cell fate at the mitochondrial level.[29,30] Bcl-2, one anti-apoptotic factor, which resides in the outer mitochondrial membrane, inhibits cytochrome c release.[30] The pro-apoptotic factor Bax resides in the cytosol. Translocation of Bax to the mitochondrial membrane might lead to loss of MMP and an increase in mitochondrial permeability. Increased mitochondrial permeability results in the release of cytochrome c from the mitochondria.[31] Released cytochrome c triggers activation of Caspase 9, which in turn activates Caspase 3; activated Caspase 3 induces cell death.

In this study we examined the modulation of the gene expression of Bcl-2 and Bax after treatment with rotenone. Our data reveal that the Bax/Bcl-2 protein ratio is increased following treatment with rotenone, but it decreases with the administration of RVS prior to rotenone. These results suggest that RVS can indeed diminish rotenone-induced apoptotic cell death. Moreover, we demonstrated that RVS prevents depolarization of MMP induced by rotenone as detected by Rhodamine 123 (Figure 5). In addition, our present findings show that rotenone induces an activation of Caspase 3, while RVS can prevent this event, supporting a strong anti-apoptotic potential for RVS. Altogether, our results demonstrate that the rotenone-induced apoptosis in SH-SY5Y cells can be prevented by RVS.

TH is the enzyme that controls the biosynthesis of dopamine, norepinephrine and epinephrin.[32] The expression of TH plays a critical role in the survival and differentiation of dopaminergic neurons. Expression of TH can be regulated by physiological and pharmacological factors. TH plays a critical role in a number of neurological disorders. For example, in PD, degeneration of the TH-positive dopaminergic cells of the substantia nigra parallels the symptoms of the disease. All successful toxin models of PD in primates and rats lead to dramatically reduced numbers of TH-positive neurons.[33,34] Meanwhile, increased activity of TH in Parkinsonian rats results in effective dopamine production and relief of disease symptoms.[35] The increase in TH expression induced by RVS treatment may therefore contribute to its neuroprotective effects.

It has been suggested that TH is subject to regulation by S-glutathionylation under conditions of oxidative stress. Neurotoxicants such as MPTP, 6-OHDA and rotenone can produce oxidative stress in neurons by various mechanisms. The damage to dopaminergic neurons associated with oxidative stress includes a reduction in dopamine as well as its biosynthetic enzyme, TH, and its high-affinity cellular transporter, DAT. Oxidative stress within dopamine neurons leads to TH S-glutathionylation via reaction of oxidizing species with GSH, and this mechanism reduces TH activity.[36] Moreover, accumulating evidence shows that α-synuclein is associated with dopamine metabolism, including the regulation of TH.[37] Environmental toxins such as MPTP and rotenone can make the α-synuclein lose its normal function via formation of α-synuclein aggregation and α-synuclein protofibrils, leading to disrupted homeostasis of dopamine metabolism and enhanced oxidative stress in dopaminergic neurons. This enhanced oxidative stress will, in turn, exacerbate the formation of α-synuclein protofibrils and drive the neuronal cells into a vicious cycle and finally cause degeneration of dopaminergic neurons.

In this study we used the human dopaminergic SH-SY5Y cell line to investigate the effect of RVS leaf extract in the rotenone model of PD. Experiments with both murine in-vitro midbrain rotenone PD models[38] and ultimately murine in-vivo models of PD[5] need to be conducted to confirm the importance of these results, gained from a human immortalized cell line. These cell lines are good tools for mechanistic studies but do not necessarily translate into neuroprotection when tested in primary or even in-vivo dopaminergic neurons.

Conclusion

This study demonstrates that RVS leaf extract protects human dopaminergic cells against rotenone-induced injury. This protection of RVS may be not only due to its ability to decrease oxidative stress-induced ROS overproduction and to inhibit apoptotic features such as changes in cellular morphology, nuclear condensation and DNA fragmentation, but also to maintenance of MMP stability, increase of Bcl-2 activity, decrease of Caspase-3 and Bax activation, as well as modulation of TH expression in SH-SY5Y cells. The RVS may therefore be an attractive candidate for the treatment of various neurodegenerative disorders associated with oxidative stress, although further research into the neuroprotective mechanisms of RVS will be necessary.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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