Protective Effects of Dodam Water Extract (Dodam) Against Rotenone-Induced Neurotoxicity in Neuro-2A Cells

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Introduction

Dodam formula (Dodam) has been used for neurodegenerative disease in Oriental medicine. Dodam is capable of protecting diverse kinds of cells from damage caused by a variety of toxic stimuli. In the present study, we investigated the underlying protective mechanism of Dodam on rotenone-induced cytotoxicity in rat neuroblastoma Neuro-2A cells. Treatment with Neuro-2A cells with rotenone caused the loss of cell viability, and condensation and fragmentation of nuclei, which was associated with the elevation of ROS level, and lipid peroxidation, the increase in Bax/Bcl-2 ratio. Rotenone induced mitochondrial dysfunction characterized by mitochondrial membrane potential loss and cytochrome-c release. These phenotypes induced by rotenone were reversed by pretreatment with Dodam. Our results suggested that major features of rotenone-induced neurotoxicity are partially mediated by mitochondrial dysfunction and oxidative stress, and that Dodam markedly protects Neuro-2A cells from oxidative injury. These data indicated that Dodam might provide a useful therapeutic strategy in treatment of the neurodegenerative diseases caused by oxidative injuries.

Key words: Dodam, Rotenone Neurotoxicity, Mitochondrial dysfunction, Neuro-2A
known as complex I) of the mitochondrial electron transport chain. Inhibition of mitochondrial respiratory chain complex I by rotenone had been found to induce cell death in a variety of cells. Rotenone is considered to exert its cytotoxicity through induction of apoptosis.14,15)

The present study, we describe the protective effect of Dodam on neuronal cell death induced by an inhibitor of mitochondrial electron transport, rotenone. We used Neuro-2A cells as a model to investigate the role of oxidative stress to neuronal apoptosis induced by rotenone. Our data demonstrate that rotenone induced mitochondrial dysfunction with ROS production, which results in apoptotic death of Neuro-2A cells.

Materials and Methods

1. Reagents

Dulbecco’s modified Eagle’s medium (DMEM) was from Gibco Life Technologies (USA). Fetal bovine serum (FBS) was purchased from Hyclone. 3-[4, 5-dimethylthiazol-2-yl]-2, 5-dephenyl tetrazolium bromide (MTT), trypsin, streptomycin, penicillin, and rotenone were obtained from Sigma. 2’-7’-Dichlorofluorescein diacetate (DCFH-DA), JC-1, and penicillin/streptomycin. Cells were maintained in DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum and 100 units/ml penicillin/streptomycin. Cells were kept at 37°C in humidified 5% CO₂ and 95% air. Experiments were carried out 24 - 48 h after cells were seeded onto plates or dishes.

4. MTT assay for cell viability

Cell viability was quantified by MTT assay. Briefly, cells were plated in 48-well culture plates at the density of 5 × 10⁴ cells/well and allowed to adhere at 37°C for 12 h. Thereafter, medium was replaced with fresh medium and cells were incubated with rotenone in the presence or absence of Dodam. After 24 h, 50 ml MTT (5 mg/mL in PBS) were added and cells incubated for an additional 4 h at 37°C. The absorbance was then measured at 595 nm using a Digiscan Micro plate Reader (Assays Hitech, Kornenburg, Austria). Wells without cells were used as blanks and were subtracted as background from each sample. Results were expressed as a percentage of control.

5. Apoptosis assay

To assess apoptosis, the nuclei of Neuro-2A cells were stained with DAPI. Cells were fixed in PBS containing 3.7% paraformaldehyde for 15 min. After fixation, cells were washed twice with PBS and then treated with DAPI (1: 5,000 dilutions) in PBS at room temperature for 15 min. After three washes, cells were observed under fluorescence microscope. To prepare genomic DNA, Neuro-2A cells (4 x 10⁴, incubated for 24 h with or without rotenone, alone, or in presence of Dodam were detached from 6 cm culture dishes and the cell suspension was centrifuged at 100 g for 10 min. The cell pellet was then washed twice with ice-cold PBS and genomic DNA was isolated using a DNA isolation kit (Promega, Basel, Switzerland) according the manufacturer’s instructions. The DNA samples was analyzed on a 1.5% agarose gel containing ethidium bromide (1 mg/mL) in TBE buffer (100 mM Tris, 90 mM boric acid, 1 mM EDTA) and run for 90 min at 70 V. After electrophoresis, the DNA was visualized under UV light and photographed.

6. Caspase activity assay

To measure the activity of caspase-3 in Neuro-2A cells, a colorimetric assay was used according to the instruction of manufacturer. Briefly, cells were treated with indicated conditions and at the indicated times, cells were washed once in cold PBS and lysed in a buffer containing 50 mM HEPES (pH 7.4), 100 mM NaCl, 0.5% CHAPS (pH 7.4), 1 mM DTT, and 0.1 mM EDTA. Cells lysate was incubated with peptide substrate in assay buffer for 2 h at 37°C. Cleavage of...
fluorogenic substrates, including 100 mM Ac-DEVD-AMC by caspase-3 was measured at an excitation of 380 nm and an emission of 460 nm. Results are represented as the percent change of the activity compared to the untreated control.

7. Determination of Mitochondrial Membrane Potential (MMP)

Mitochondrial membrane potential was quantified using the ratio metric probe JC-1 (Molecular Probes, USA). In healthy Neuro-2A cells, the intact membrane allows the lipophilic dye JC-1 to enter into the mitochondria where it accumulates and aggregated, producing an intense orange signal. In apoptotic cells, where the mitochondrial membrane potential has collapsed, the monomeric JC-1 remains in the cytosol and appears green. Neuro-2A cells were treated for 15 h with 500 nM rotenone in the absence or presence of Dodam. Cells were incubated for 30 min with 10 mg/ml JC-1 at 37°C under 5% CO2, and then washed twice with 0.1 M PBS. The proportion of aggregated vs. monomeric JC-1 probe was visualized under UV light and photographed.

8. Mitochondrial complex I activity (NADH - ubiquinone oxidoreductase assay)

Mitochondria complex I activity was determined by the method of Ian et al.,12, which followed the decrease in NADH absorbance at 340 nm that occurred when ubiquinone (CoQ1) was reduced to form ubiquinol17). The reaction was initiated by adding CoQ1 (50 mM) to the reaction mixture, at 30°C. After 5 min, rotenone was added and the reaction was further registered for 5 min. Complex I activities were expressed in nanomoles per minute per milligram of protein and represent the rotenone sensitive rates.

9. Western blot analysis

Cytosolic protein extracts was prepared as previously described16). Briefly, cells were collected by centrifugation at 300 x g for 5 min at 4°C and washed with ice-cold PBS. The cell pellet was then resuspended in 500 ml of lysis buffer (20 mM Hepes- KOH, pH 7.5, 210 mM sucrose, 70 mM mannitol; 1.5 mM MgCl2, 10 mM KCl, 10 ug/ml leupeptin, and 10 mM digitonin). After 10 min incubation at 25°C, the sample was spun at 14,000 x g for 15 min, and the supernatant containing cytosolic proteins was stored at -70°C until analysis by polyacrylamide gel electrophoresis (SDS-PAGE). The protein extract was subjected to standard SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Millipore), and probed with appropriate antibodies as described individually in the figure legends. The bound primary antibody was detected using appropriate horseradish peroxidase-conjugated secondary antibody, and protein was visualized using an enhanced chemiluminescence detection kit. β-actin was used as an internal control to confirm that the amounts of protein load were equal.

10. Detection of cytochrome-c release

Cytochrome-c released from mitochondria was determined by Western blot analysis. Cytosolic and mitochondrial fractions were prepared as below, but the fractionation buffer contained 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl2, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 20 mM HEPES, pH 7.0, and protease inhibitors (1 mM sodium orthovanadate, 10 mg/ml leupeptin, 10 mg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The mitochondrial pellet was resuspended in the same buffer, sonicated, and centrifuged to obtain the soluble fraction. Equal amounts of protein were separated on a 10% SDS polyacrylamide gel and transblotted onto polyvinylidene difluoride-nitrocellulose filters. Specific cytochrome-c band was detected by using anti-cytochrome-c antibody (1:500) followed by enhanced chemiluminescence-based detection.

11. Measurement of intracellular ROS

ROS generation in cells was measured by using fluorescent probes 2′, 7′-Dichlorofluorescein diacetate (DCFH-DA, Molecular Probes, Eugene, OR, USA) and hydroethidium (HE). Cells in 96-well plates were incubated for 24 h, reacted with 10 mM DCF-DA at 37°C for 30 min, and washed twice with PBS. Fluorescence intensity of DCF was measured by using microplate-reader (Fluoroskan Ascent Thermo Labsystems) at excitation wavelength 485 nm and emission wavelength 538 nm. Blue fluorescent dihydroethidium is oxidized to red fluorescent ethidium in the presence of superoxide. Therefore, the ethidium fluorescence intensity is proportional to the amount of superoxide formed intracellularly. Cells were washed twice with PBS and incubated in Krebs Ringer HEPES solution containing 5 mM HE at 37°C for 30 min. The fluorescence was measured by flow cytometry (FACSCalibur, BD Biosciences).

12. Lipid peroxidation (MDA-TBA assay)

Lipid peroxidation assay was based on that described by Ohkawa et al.,20 with some modifications reported by Ford et al.,21. Briefly, 50 ml of PBS suspension of harvested cells (1 × 10⁶ cells) were added 50 ml of 3.2% SDS in PBS, and incubated for 10 min at room temperature. Following incubation, 150 ml of 20% acetic acid (pH 3.5) and 150 ml of 0.8% TBA (thiobarbituric acid) in 0.05 N NaOH were added and the mixture was then boiled for 1 h. The mixture was
cooled and extracted with 1 ml each of n-butanol-pyridine mixture (1:3, v/v) to avoid turbidity. The upper layer (approximately 240 ml) of each sample was aspirated, and the absorbance was measured at 532 nm. Concentration of 2-TBA was determined using the extinction coefficient of $1.56 \times 10^5$ M$^{-1}$cm$^{-1}$. The results were expressed as mol of malondialdehyde (MDA) per mg-protein. Protein was determined by the method of Lowry using bovine serum albumin as a standard.

13. Statistical analysis
The data given in the text are expressed as mean ± S.D. Statistical comparison was carried out with three or more groups using one-way analysis of variance (ANOVA). A difference was considered to be significant at P < 0.05.

Results
1. Treatment with Dodam protected Neuro-2A cells from Rotenone-induced cytotoxicity
We tested the cytoprotective effects of Dodam on rotenone-induced toxicity in Neuro-2A cells. First, we evaluated the cytotoxicity of rotenone in Neuro-2A cells. As shown in Fig. 1A, cells were exposed to increasing doses of rotenone for 24 h and cell viability was determined MTT assay. Cell viability was markedly decreased 24 h after rotenone treatment in a dose-dependent manner. However, when cells were pretreated with Dodam (0.125 - 1.5 mg/ml), rotenone-induced cytotoxicity was significantly attenuated (Fig. 1B). The protective effect of Dodam was dose-dependent manner with a maximal effects being reached around 1 mg/ml. Thus, Dodam alone seems to be did not cause any cytotoxic effect on the cell viability.

2. Effects of Dodam on Rotenone-induced apoptosis in Neuro-2A cells
To characterize whether the cell death by rotenone was caused by apoptosis, typical assay was performed. First, morphological change of apoptosis was assessed using DAPI staining. As shown in Fig. 2A-a, the nuclei of control cells had a regular and ovum shape. However, apoptotic nuclei characterized by nuclear condensation and fragmentation, appeared after exposure to 0.5 mM rotenone for 24 h (Fig. 2A-b). Dodam (0.5 mg/ml) pretreatment blocked the rotenone-induced nuclear damage, while the nuclear morphology of cells exposed to Dodam alone was similar to that of untreated cells. Second, we observed DNA fragmentation using agarose electrophoresis. Cells treated with rotenone showed a ladder pattern of discontinuous DNA fragments (Fig. 2B-b). However, Dodam prevented DNA fragmentation by rotenone (Fig. 2B-d).

We also evaluated the activation of caspase-3 in cellular extract of Neuro-2A cells using fluorometric assay as a hallmark of apoptosis. The effect of Dodam on rotenone-induced caspase-3 activation was shown Fig. 2C. Following 24 h treatment with rotenone, we detected a caspase-3 activity increase to 2.5 folds of the control level. Pretreatment with Dodam significantly attenuated rotenone-induced caspase-3 activation. Dodam alone did not...
show a significant effect on the caspase-3 activity in Neuro-2A cells, which was consistent with its lack of apoptotic response. 

3. Treatment with Dodam prevented mitochondrial dysfunction in rotenone-treated Neuro-2A cells

The interruption of electron flow at complex I can lead to reactive oxygen species (ROS) production due to redox cycling of reduced electron carriers upstream of the inhibition site in mitochondria. If rotenone was acting at complex I to inhibit bioenergetics in Neuro-2A cells, we predict that this action would eventually lead to mitochondrial membrane permeability transition (MMPT), since MMPT can be initiated by ROS. We first examined the effect of rotenone-induced cell death is mediated via mitochondrial function. A mitochondrial activity marker JC-1 forms monomers of green fluorescence at a low membrane potential, which becomes J-aggregates of orange fluorescence at a higher membrane potential. Fig. 3A revealed that Dodam prevented the rotenone-induced MMPT, while treatment of rotenone resulted in a disruption of MMPT (Fig. 3A-b).

The mitochondrial membrane transition pore opening is associated with collapse of the membrane voltage, resulting in the release of cytochrome-c into cytosol. Using Western blotting, second, we investigated the possible effects of Dodam on the rotenone-induced cytochrome-c release from mitochondria. As shown in Fig. 3B, rotenone could induce cytochrome-c release significantly. While cytochrome-c protein levels in the cytosolic fraction of the cells treated with Dodam or Dodam plus rotenone for 24 h showed a slightly increase, the protein level was markedly increased in cells treated with rotenone only (Fig. 3B).

The Bcl-2 family consists of both apoptotic and anti-apoptotic proteins and the balance between these proteins is critical to turning on and off the cellular apoptotic machinery. Bcl-2 family members are intimately involved in cell death processes caused by rotenone. In this study, we investigated whether Dodam has any effect on the expression of Bax and Bcl-2 in rotenone-treated cells using Western blot analysis. As shown in Fig. 3C, Bax protein expression was significantly increased in rotenone-treated cells compared with control cells. However, pretreatment with Dodam (0.5 mg/ml) could decrease the level of Bax expression almost to the normal value. These results suggested a notion that Dodam pretreatment shifted the balance between positive and negative regulators of apoptosis towards cell survival.

In additionally, Complex I and complex III activities were measured in all preparations of mitochondria. As shown in Table 1, mitochondria from rotenone treated Neuro-2A cells exhibited a marked decrease in the activity of both enzyme complexes, including complex I and complex III, compared with control and Dodam only treated groups. However, Dodam pretreatment had significant protective effects and actually attenuated the decline in the complex I and complex III activities.

4. Treatment with Dodam suppressed the generation of ROS by rotenone in Neuro-2A cells

Rostenone interacts with mitochondrial complex I,
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blocking ATP production and promoting oxygen free radical formation. To determine whether Dodam could prevent the rotenone-induced ROS generation and the resulting oxidative stress, levels of ROS production in cells was determined using the fluorescence probes, DCFH-DA and hydroethidium (HE). Cells exposed to rotenone for 15 h displayed the increased intensity of DCF-labeled cells compared to control cells. The addition of rotenone to Neuro-2A cells caused a 2-folds increase in the DCF fluorescence. However, cells pretreated with Dodam showed a reduced intensity of DCF-labeled cells when compared to rotenone-treated cells (Fig. 4A). Fig. 4B demonstrated a typical flow cytometric histogram of hydroethidium. The red fluorescence detected in rotenone-treated cells indicated that rotenone indeed caused the generation of superoxide (O$_2^-$). Cells pretreated with Dodam markedly suppressed the intracellular superoxide level induced by rotenone. The results suggested the involvement of ROS in rotenone-induced cytotoxicity and anti-oxidative activity of superoxide.

5. Treatment of Dodam inhibited the lipid peroxidation by rotenone in Neuro-2A cells

To determine whether the observed increase in the rate of lipid peroxidation in the Neuro-2A cells treated with rotenone, we assessed lipid peroxidation by using TBARS assay. Although cells treated with rotenone for 6 h showed no significantly differences in lipid peroxidation according to increasing concentrations of rotenone, when cells were treated with rotenone for 24 h, the rate of lipid peroxidation significantly increased in a dose and time dependent fashion (Fig. 5A). As shown in Fig. 5B, treatment with rotenone for 24 h resulted in showed almost two folds of TBARS formations with respect to control or Dodam only treated cells.

Discussion

The aim of the present investigation was to evaluate the protective effect and to determine the possible molecular mechanisms of Dodam in rotenone-treated Neuro-2A cells. In this study, we demonstrated that Dodam protects Neuro-2A cells against rotenone-induced cytotoxicity through inhibition of mitochondrial dysfunction and activation of caspase cascades. Mitochondria are considered as a critical mediator of cell death in various neurodegenerative diseases. Especially, mitochondrial dysfunction caused by reduced activity of either complex I or complex III is known to be associated with traumatic brain injury and a number of neurodegenerative diseases. These electron transport chain dysfunctions can be mimicked using specific blockers, such as rotenone. Complex I can be specifically blocked by rotenone and complex III is blocked by antymycin. Treatment with these blocking agents prevents transfer of electrons down the chain but at the same time accentuates their ability to transfer electrons to oxygen and generate superoxide.

Rotenone interacts with mitochondrial complex I, blocking ATP production and promoting the formation of
oxygen free radicals [31]. The mitochondrial derived ROS are vital not only because mitochondrial respiratory chain components are present in almost all eukaryotic cells, but also because the ROS produced in mitochondria can readily influence mitochondrial function without having to cope with long diffusion times from the cytosol. Consistently with these reports, several lines of evidence suggest that the mitochondrial membrane potential may be an effector of both the release of cytochrome c and the induction of Bcl-2/Bax [14]. The loss of mitochondrial membrane potential is related to the release of apoptogenic molecules, including cytochrome-c and activation of the pro-apoptotic proteins near from the outer mitochondrial membrane. Our present results shown that rotenone induced mitochondrial dysfunction, release of cytochrome-c, down-regulation of Bcl-2, and up-regulation of Bax protein in Neuro-2A cells. It also demonstrated that rotenone increased oxidative damages, including lipid peroxidation, in Neuro-2A cells and that the cellular redox status are critically important in determination of susceptibility of these cells against inhibition of mitochondrial complex I. However, Dodam markedly suppressed rotenone-induced ROS generation, which may be one of the underlying mechanisms of neuro-protective effects of Dodam. In conclusion, the present study demonstrates that Dodam herbal formula may have clinical advantages in treatment of neurodegenerative disorders caused by perturbation of redox homeostasis with oxidative stresses.

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Reference


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