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RESEARCH ARTICLE

Investigation of the antioxidant activity of hesperidin against 6-hydroxydopamine-induced cell damage in SH-SY5Y cells

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ABSTRACT

Background: Parkinson's disease (PD) is regarded as a neurological condition in which continuous degeneration of dopaminergic neurons occurs selectively. Currently, most treatments for neurodegenerative diseases are palliative. According to *in vitro* and *in vivo* models of PD in recent studies, hesperidin (Hsd) showed protective properties during neuron damage. Moreover, recent reports demonstrated the induction of Hsd. **Aims and Objectives:** The current study aimed at analyzing the protective effect of Hsd, as a major flavanone constituent by determining its effect on 6-hydroxydopamine (OHDA)-mediated oxidative stress. The current study analyzed the impact of Hsd on neurotoxicity, mediated by 6-OHDA, in SH-SY5Y cells by an *in vitro* model of PD. **Materials and Methods:** The study employed 150 μM of 6-OHDA to induce cellular damage. Furthermore, 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide test was performed to analyze cellular viability. Fluorescence spectrophotometry was performed to measure the level of intracellular reactive oxygen species (ROS) and intracellular calcium. Based on the findings, 6-OHDA could reduce cell viability. **Results:** Moreover, intracellular ROS, intracellular calcium, and DNA fragmentation vastly improved in cells exposed to 6-OHDA. SH-SY5Y cell incubation with Hsd (1 and 10 μg/mL)-induced protective effects and decreased the biochemical markers of cell apoptosis. According to the findings, Hsd showed protective features against neurotoxicity, caused by 6-OHDA. These protective properties were accompanied by anti-apoptotic features. **Conclusion:** It was revealed that Hsd affected the management of PD. Given the preserved mitochondrial function of Hsd , and its antioxidant and anti-apoptotic properties in neuroblastoma cell lines, this compound has neuroprotective effects on 6-OHDA.

KEY WORDS: Hesperidin; Parkinson's Disease; 6-Hydroxydopamine; Apoptosis

INTRODUCTION

Parkinson's disease (PD) is recognized as the second most prevalent neurological condition worldwide. Although the cause of PD was clarified, several studies showed that in individuals with PD, the *Substantia nigra* displayed oxidative contents such as iron, dopamine, and neuromelanin. Low antioxidant content is vulnerable to oxidative damage.[1] According to the literature, variations in calcium homeostasis, inflammation, activation of apoptotic factors, and abnormalities in the ubiquitin-proteasome system characterize PD progression. In 1994, Mattson was the first to show that 6-hydroxydopamine (6-OHDA) had an apoptotic effect. It is one of the most common neurotoxic substances used to analyze PD progression both in the *in vivo* and *in vitro*.[2]

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Current pharmacological therapies for PD have several side effects. Herbal medicines are widely perceived as natural and safe. Citrus fruits and their products are widely consumed worldwide as seasoning, food, and medicine. [3] Hesperidin (Hsd), a flavone glycoside of citrus fruit, exists in citrus and other plants and can be isolated in large amounts from the peels of *Citrus aurantium* (bitter orange), *Citrus sinensis* (sweet orange), and *Citrus unshiu* (satsuma mandarin). It is reported that Hsd has a wide range of antioxidant, anti-inflammatory, antifungal, antiviral, and anticarcinogenic properties.[4,5]

Some *in vitro* and *in vivo* studies revealed the antioxidant and free-radical-scavenging features of Hsd.[6] Based on these models, Hsd had neuroprotective features. For instance, in previous studies, Hsd demonstrated neuroprotective effects on amyloid-β [7] and neurotoxicity mediated by 3-nitropropionic acid[8] and hydrogen peroxide.[9]

The control of intracellular reactive oxygen species (ROS), calcium homeostasis, and the caspase cascade involved in apoptosis is among new strategies to prevent and treat PD. With this background in mind, the current study analyzed the protective effects of Hsd , as a major flavanone constituent, by determining its effect on 6-OHDA-mediated oxidative stress generation, mitochondrial dysfunction, and apoptosis in human neuroblastoma SH-SY5Y cells.

MATERIALS AND METHODS

Substances

The following substances were provided by Sigma-Aldrich Co. (MI, USA): Hsd , DADLE, fura-2-acetoxymethyl ester (fura-2-AM), 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT), 6-OHDA, 2,7-dichlorofluorescein diacetate (DCFH-DA), and rhodamine 123. Furthermore, Dulbecco's modified Eagle's medium (DMEM) purchased from Sigma. The primary antibodies, including polyclonal anti-caspase-3 and monoclonal anti-β-actin, were provided by Cell Signaling Technology Co. (MA, USA).

Primary monoclonal anti-cytochrome c antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, USA); horseradish peroxidase (HRP) secondary antibody conjugates from GE Healthcare Bio-Sciences (NJ, USA); the culture reagents, trypsin-ethylenediaminetetraacetic acid (EDTA), fetal bovine serum, and the solution containing penicillin and streptomycin from Biocera (East Sussex, UK), and culture flasks and dishes from SPL Life Sciences (South Korea).

Cell Culture

SH-SY5Y cell line was supplied by the National Cell Bank of Iran, established at Pasteur Institute (Tehran, Iran). DMEM, supplemented with 100 μg/mL of streptomycin, and 100 U/mL of penicillin, was used to grow the cells; the medium was changed every 2 days. A CO_2 incubator was used to maintain the cells at a temperature of 37°C in an atmosphere with saturated humidity (95% air, 5% CO2). Before each experiment, dimethyl sulfoxide (DMSO) was used to dissolve 6-OHDA and Hsd .

In Experiment I, various 6-OHDA concentrations (50, 100, 150, 200, and 250 μM) were used to incubate the cells for 24 h, and MTT assay was performed to detect the IC50 value of 6-OHDA. In Experiment II, cells were pretreated with different concentrations of Hsd (1, 10, 100, and 1000 μg/mL)for 24 h and, then, incubated with 6-OHDA (effective dose) for 30 min. A 96-well plate (5000 cell/well) was used to seed the cells for intracellular calcium, MTT, and ROS assays. Furthermore, to grow the cells in Western blot analysis, a 6-well plate was used for 24 h.

Analysis of Cell Viability

The analysis of cell viability was based on the reduction of MTT to formazan. The wells contained MTT at a final concentration of 0.5 mg/mL. Incubation was performed at 37°C over 2 h. Afterward, the medium was removed and DMSO (100 μL) was added to each well. Absorbance at 490 nm was measured in an enzyme-linked immunosorbent assay (ELISA) MAT 2000 microplate reader (DRG Instruments, Marburg, Germany). The percentage of control cells was measured to present the findings.[10]

Determination of Intracellular ROS Production

A fluorescent probe (DCFH-DA) was used to analyze ROS production. DCFH-DA can turn into dichlorofluorescein, which is highly fluorescent, by a proper oxidant. Incubation was performed using DCFH-DA (1 mM) and phosphate-buffered saline (PBS) in a dark chamber at a temperature of 37°C in 10 min. Following that, PBS was used to rinse the cells 3 times; the cells were assessed instantly, using the fluorescence plate reader (FLx800, BioTek, USA). The cells were examined in terms of fluorescence intensity in plates with excitation and emission filters of 485 and 538 nm, respectively; it should be noted that each test was performed 6 times. To present the findings, the fluorescence percent was measured to control cells.[6]

Measurement of Intracellular Calcium [Ca2+]

In 60 min, the cell groups were filled with fura-2-AM (5 μM). Then, fura-2-AM was rinsed and removed, and dye-free saline was used to incubate the cell cultures. The fluorescence plate reader was employed to assess the cells immediately. The fluorescence intensity of cells was analyzed in the 96-well plate with excitation and emission filters (340/380 and 510 nm, respectively). It should be noted

that every experiment was performed 6 times separately. To express the findings, the fluorescence percent was measured in the control cells.[6]

Western Blot Analysis

To detect cytochrome c and cleaved caspase 3, cellular lysis was performed in 30 min on an ice lysis buffer consisting of the following agents: 20 mM of HEPES (pH 7.6), glycerol (20%), 500 mM of NaCl, 1.5 mM of $MgCl_2$, 0.2 mM of EDTA, Triton X100 (0.1%), 10 μg/mL of aprotinin, 2.5 μg/mL of leupeptin, 0.5 mM of phenylmethanesulfonyl fluoride, 1 mM of dithiothreitol, and sodium dodecyl sulfate (SDS).

Afterward, separation of lysates was carried out at 14,000 g during 30 min, and the supernatants were eliminated and the pellets resuspended in 50 μL of buffer[11] to detect cytochrome c, cleaved caspase 3, and beta-actin. Homogenization of the cells was performed in an ice-cold buffer consisting of the following agents: EDTA (1 mM), Tris-HCl (10 mM), 0.1% SDS, 0.1% sodium deoxycholate, sodium orthovanadate (1 mM), and 1% NP40 with protease inhibitors (1 mM, 10 μg/mL, and 2.5 μg/mL of phenylmethanesulfonyl fluoride, aprotinin, and leupeptin, respectively).

Homogenate centrifugation was carried out at 14,000 rpm in 15 min at 4°C. The final supernatant was maintained as the whole cell fragment. The Bradford technique was used to analyze the protein. SDS-polyacrylamide gel electrophoresis (9%) was performed for the electrophoretic measurement of equivalent amounts of protein. Polyvinylidene difluoride membranes were the target of proteins (GE Healthcare Bio-Sciences, NJ, USA). Probing was performed with rabbit monoclonal caspase-3 antibody (1/1000 dilution overnight at 4°C) for 3 h at a temperature of 37°C. Before that, blocking was done with dried milk (fat-free) in Tris-buffered saline (TBS) and 0.1% Tween 20 (containing 150 and 20 mM of NaCl and Tris-HCl, respectively; pH 7.5) at 4°C.

After washing (3 times, 5 min each) in TBS, and supplemented with 0.1% Tween 20, the blots were kept at room temperature for 60 min with a HRP secondary antibody conjugate (1/15,000 dilution). Blocking buffer was used to extenuate the antibodies. To differentiate complexes of antibodies and antigens, the ECL system was used. Then, Lumi-Film Chemiluminescent Detection Film (Roche, Germany) was used to expose the complexes. The degree of release was determined by LabWorks™ (UVP, UK). Furthermore, β-actin (1/1000 dilution) was used as the loading control.

Statistical Analysis

The mean ± standard error of mean (SEM) for the four experiments was measured to present the findings of each group. ANOVA and the Newman-Keuls test were performed for intergroup comparisons. SPSS version 18.0 was applied, and the significance level was set at 0.05.

RESULTS

Protective properties of Hsd regarding cell viability 6-OHDA treatment (50, 100, 150, 200, and 250 μM for 24 h) of SH-SY5Y cells induced approximately 50% dose-dependent reduction in cell proliferation at 150 μM [Figure 1a]. Hsd doses dependently (1, 10, 100, and 1000 μg/mL) attenuated the changes in cell proliferation induced by 150 μM 6-OHDA [Figure 1b], with approximately 85% protection following treatment with 1 and 10 μg/mL after 24 h.

A significant toxic effect was observed in cells treated with 150 μM of 6-OHDA [Figure 1a]. Incubation of 1 and 10 μg/mL of Hsd could significantly prevent cell toxicity mediated by 6-OHDA ($P < 0.05$) [Figure 1b].

Effect of Hsd on 6-OHDA-mediated ROS Generation

ROS generation SH-SY5Y cells exposed to 150 μM of 6-OHDA were measured by fluorescence spectrophotometry, using DCFH-DA (excitation 528/20 nm, emission 528/20 nm). As shown in Figure 2a, an evident rise in DCF signaling and ROS generation was observed in cells incubated with Hsd (1, 10, 100, and 1000 μg/mL), compared to the ones incubated with the control cultures. At 2 doses, 1 and 10 μg/mL, the corresponding values significantly declined, compared to the 6-OHDA-treated group [Figure 2b].

Determination of Intracellular Calcium in SH-SY5Y Cells

The intracellular calcium level significantly ($P <0.001$) improved in the 6-OHDA-treated group. Furthermore, 1 and 10 μg/mL of Hsd could significantly ($P <0.05$ and <0.001, respectively) suppress 6-OHDA-induced intracellular calcium elevation [Figure 3].

Influence of Hsd on Cleaved Cytochrome c and Activated Caspase-3 in SH-SY5Y Cells

Cytochrome c release activation was analyzed to find the effect of Hsd agonists on possible mediators of cell apoptosis caused by 6-OHDA. Incubation of SH-SY5Y cells was performed using 6-OHDA either independently or along with Hsd (1 and 10 μg/mL). According to Figure 4a, cell treatment using 150 μM of 6-OHDA significantly elicited Hsd and could significantly inhibit the release of cytochrome c.

In addition, cleaved caspase-3 elevated following 6-OHDA treatment ($P <0.001$). Hsd doses of 1 and 10 μg/mL could decrease 6-OHDA-mediated activation of cleaved caspase-3 [Figure 4b].

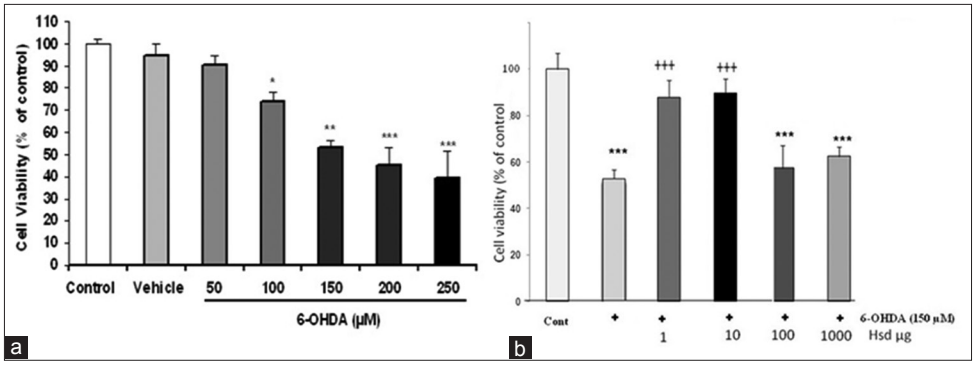

Figure 1: The dose-dependent effect of 6-hydroxydopamine (6-OHDA) on cell viability after 24 h (a), **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 compared to the control group. The effect of different doses of hesperidin (1, 10, 100, and 1000 μg/mL) alone and against 6-OHDA rotenone-induced changes on cell proliferation (b), values are expressed as mean ± standard error of mean, *n* = 5–6 wells for each group; ****P* < 0.001 compared to the control group and +++ *P* < 0.001 compared to the control group

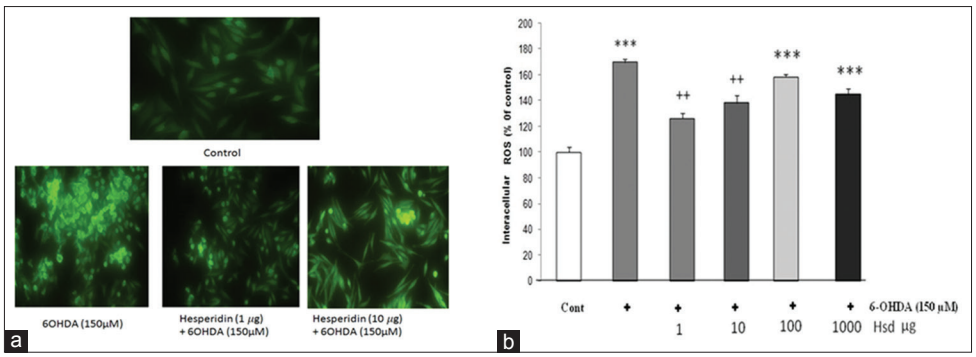

Figure 2: Microscopic images showing the preventive effects of hesperidin against rotenone-induced reactive oxygen species (ROS) generation by DCFDA staining (a). The effects of hesperidin on ROS production in SH-SY5Y cells (b), values are given as mean ± standard error of mean, *n* = 5–6 wells for each group. ****P* < 0.001 versus the control cells, ++*P* < 0.01 versus 6-OHDA-treated cells

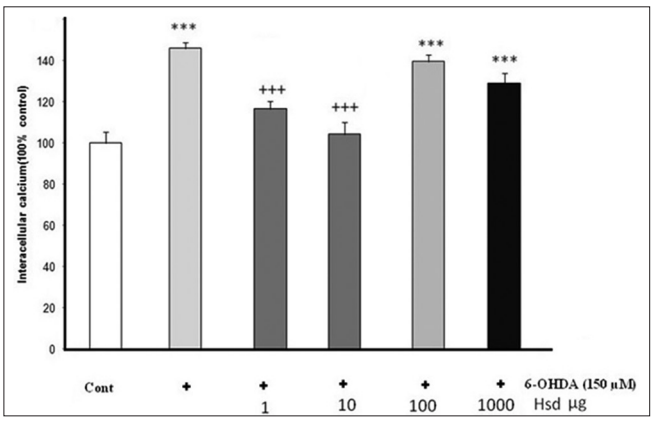

Figure 3: The effect of hesperidin on 6-hydroxydopamine (6-OHDA)-induced intracellular calcium elevation; values are expressed as mean ± standard error of mean, *n* = 5–6 wells for each group. ****P* < 0.001 versus the control cells, +++*P* < 0.01 versus 6-OHDA

DISCUSSION

The current study findings confirmed the cytotoxic effect of 6-OHDA on SH-SY5Y cells, which aligned with the results of previous studies.[11] The current study assessed the activity of Hsd against 6-OHDA-mediated cytotoxicity. Based on the findings, different mechanisms, separately or concomitantly, might contribute to the neuroprotective effects of Hsd . However, the molecular mechanisms that underlie Hsd 's neuroprotective effects are not fully understood. The current study showed that treatment with 6-OHDA resulted in a significant increase of ROS and calcium level.[11] Overproduction of ROS can induce severe damage to cellular action. Roohbakhsh *et al*. showed that ROS was in the apoptotic mechanisms of 6-OHDA mediation, rotenone activity, and β-amyloid neurotoxicity.[12] Some antioxidants prevent cell apoptosis in rotenone-treated dopaminergic neuronal cell lines.[13] Previous studies suggested that Hsd had stronger antioxidative activity,[9,12] with respect to the reduction of 6-OHDA-mediated ROS production and the increase in cell apoptosis and mitochondrial damage (caused by 6-OHDA) in SH-SY5Y cells.[14]

ROS generation has negative effects on the potential of mitochondrial membranes and leads to the expression of apoptotic factors and activation of cytochrome c and caspase-3 cascade.[15] The data showed that cytochrome c releases from mitochondria in 6-OHDA-treated cells, findings that align with those of previous studies. Caspase-3 protein contributes to the apoptotic process in two ways.[16] The DNA fragmentation factor is activated by death receptor and mitochondrial apoptotic pathway caspase-3. This process

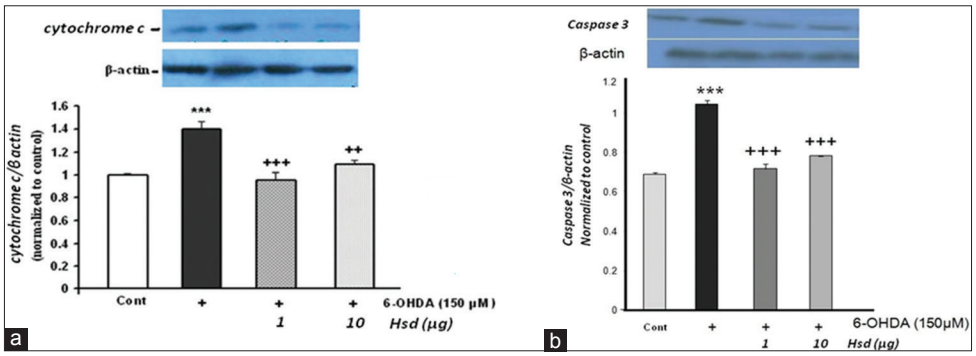

Figure 4: The effect of hesperidin on the 6-hydroxydopamine-induced cytochrome c (a) and caspase-3 (b) released in SH-SY5Y cells; values are presented as mean ± standard error of mean band density ratio for each group. β-actin was used as an internal control; ++$P < 0.01$, +++$P < 0.001$, and ***$P < 0.001$ compared to the control cells

stimulates endonuclease cleavage of nuclear DNA and results in apoptosis. Various factors promote mitochondrial permeability transition and cytochrome c expression in the mitochondrial pathway, thereby activating caspase-3. In the current research, 6-OHDA increased caspase-3 activity; this finding was in line with the results reported by Song *et al*.[17] In a previous study, Hsd cotreatment successfully inhibited cytochrome c and caspase-3 activities, caused by 6-OHDA.[18] Based on the current study results, Hsd may act upstream of caspase-3 to block apoptosis.

The DNA fragmentation factor is cleaved by the active caspase-3. DNA fragmentation, as a result of inhibition of 6-OHDA in Hsd incubated SH-SY5Y cells, was also observed by Hwang and Yen.[19] In addition, other possible mechanisms, including the potential of Hsd to reduce the calcium content, could be involved in its protective effect on 6-OHDA-induced oxidative stress.

According to the recent research, increase in calcium entry is consistently linked to neurotoxin-mediated degeneration of neurons and cell damage. Cellular and animal models of PD demonstrated changes in intracellular calcium homeostasis to increase Ca^{2+}.[19] In a recent study, Wang *et al*. revealed that PD is related to changes in the release of Ca^{2+} channels and binding proteins; in fact, it causes particular neurons to be more susceptible to degeneration of neurons.[20]

It is also shown that in SH-SY5Y cells, β-synuclein increases Ca^{2+} level and seriously harms the neurons due to Ca^{2+} homeostasis deregulation. Intracellular calcium rise and neurodegeneration were inhibited by preliminary treatment with Hsd in SH-SY5Y cells. Hsd could couple with an L-type Ca^{2+} channel and thus exhibit Ca^{2+} channel-blocking activity.[20] Nevertheless, this potential mechanism should be further analyzed; it should be noted that Hsd might block the Ca^{2+} channel. Although the exact mechanism behind the neuroprotective effects of Hsd remains unclear, it is likely to demonstrate both antioxidant and calcium blocking properties. The data obtained by the study suggested that Hsd therapy could significantly attenuate ROS formation. It is necessary to perform more detailed research on the neuroprotective mechanisms of Hsd before making a decision concerning the effect of Hsd on the treatment of PD and similar neurodegenerative diseases.

CONCLUSION

The current study findings might provide a new clinical insight into progressive neurodegenerative diseases such as PD.

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