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Original Research Article

Hesperetin protects SH-SY5Y cells against 6-hydroxydopamine-induced neurotoxicity via activation of NRF2/ARE signaling pathways

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Abstract

Purpose: *To investigation the protective effects of hesperetin against 6-hydroxydopamine (6-OHDA)-induced neurotoxicity.*
Methods: *SH-SY5Y cells were incubated with 6-OHDA to create an in vitro model of neurotoxicity. This model was used to test the neuroprotective effects of hesperetin. Cell viability was assessed by MTT and lactate dehydrogenase (LDH) release assays. Flow cytometry and western blot were used to quantify apoptosis. Oxidative stress was evaluated by determining intracellular glutathione (GSH), malondialdehyde (MDA), superoxide dismutase (SOD), and reactive oxygen species (ROS).*
Results: *In SH-SY5Y cells, treatment with 6-OHDA decreased cell viability and promoted LDH release. However, exogenous hesperetin protected against 6-OHDA-mediated toxicity. Similarly, although incubation with 6-OHDA induced apoptosis and increased cleaved caspase-3 and -9 levels, treatment with hesperetin protected against these effects. Treatment with 6-OHDA also led to significant oxidative stress, as indicated by reduced GSH and SOD levels and increased MDA and ROS levels in SH-SY5Y cells. However, these changes were reversed by pre-treatment with hesperetin. Of interest, hesperetin led to changes in 6-OHDA-induced expression of NRF2, heme oxygenase-1 (HO-1), glutamate-cysteine ligase (GCL) catalytic subunit (GCLC), and GCL modulatory (GCLM).*
Conclusion: *Hesperetin protects against cell toxicity, apoptosis, and oxidative stress via activation of NRF2 pathway in a 6-OHDA-induced model of neurotoxicity. Future studies should investigate the use of hesperetin as a potential therapeutic approach for prevention or management of Parkinson's disease.*

Keywords: *Hesperetin, 6-OHDA, Neurotoxicity, NRF2, Parkinson's disease*

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INTRODUCTION

Parkinson's disease (PKD) is a nerve degenerative disease without effective neuroprotective treatment [1]. However, there is currently no treatment for PKD. Improper balance between the intracellular antioxidant and oxidation systems contributes to the development of PKD [2]. Of note, NRF2 protects against oxidative neurological stress [3] and

plays a central role in the biology of PKD. NRF2-mediated transcriptional deletion increases the susceptibility of dopaminergic neurons to oxidative stress during PKD [4], and mice that are deficient in NRF2 are more susceptible to toxin-mediated damage of neurons in the substantia nigra than wild-type mice [5]. Moreover, activation of NRF2 leads to neuroprotection against toxins [6,7]. Therefore, NRF2 seems to be a potentially valuable target for new therapies aimed to treat or prevent PKD.

Hesperidin is a common, inexpensive plant flavonoid that is derived from citrus plants, such as sweet oranges and lemons [8]. Hesperidin has many pharmacological properties, including anti-hypercholesterolemia, anti-inflammatory, antioxidant, and neuroprotective effects [8]. Hesperetin is an aglycone of hesperidin that is produced by the intestinal microflora and has been shown to have anticancer effects in a mouse model of prostate cancer [9]. Recently, hesperidin was reported to prevent H_2O_2-induced oxidative damage by upregulating the NRF2 pathway [10]. Important to the topic of this work, hesperetin has been shown to suppress oxidative stress and protect against Alzheimer's disease [11]. However, the potential benefits and mechanisms of hesperetin in the treatment of PKD remain poorly understood to date.

In this study, we established a 6-OHDA-induced cell model of PKD to evaluate the therapeutic potential of hesperetin and the role of the NRF2 signaling pathway in PKD.

EXPERIMENTAL

Cells and treatment

Cells were cultured in DMEM-F12 medium (Lonza, Basel, Switzerland) with 10 % FBS (Gibco, Gaithersburg, MD, USA) in a constant temperature incubator. Cells were treated with 10, 20, or 50 μM hesperetin for 2 h and then treated with 100 μM 6-OHDA for 24 h.

Evaluation of cell viability

Cells (1×10^3 per well) were incubated with 5 mg/mL of MTT for 4 h. After removing the media, cells were incubated and the absorbance was read at 490 nm.

Assessment of lactate dehydrogenase (LDH) release

After incubation with hesperetin and 6-OHDA, 100 μL of cell culture medium was incubated with LDH reaction reagent for measurement of LDH release.

Flow cytometry

Cells were harvested and resuspended in binding buffer with R-phycoerythrin (PE; 5 μL, 100 μg/mL) and ribonuclease (1 U/mL). The cells were incubated with 5 μL of FITC-conjugated Annexin V before analyzed by FACS flow cytometry (Attune; Life Technologies, Darmstadt, Germany).

Measurement of oxidative stress

After treatment with 10, 20, or 50 μM hesperetin for 2 h and incubation with 100 μM 6-OHDA for 24 h, cells were harvested, and levels of MDA, SOD, GSH, and ROS were determined using commercially available kits (Beyotime Biotechnology, Shanghai, China).

Western blot assay

Cell proteins were extracted and total protein concentrations were determined via BCA assay. Proteins were separated by SDS-PAGE and then transferred onto a PVDF membrane, which was then incubated with primary antibodies against cleaved caspase-3, cleaved caspase-9, NRF2, HO-1, GCLM, GCLC, and GAPDH (Abcam, Cambridge, MA, USA) followed by a secondary antibody. Relative densitometries were analyzed using Image J software and compared to GAPDH as a loading control.

Statistical analysis

The results were shown as mean ± SD. Graph Pad Prism 5 was used to conduct one-way analysis of variance. Comparisons resulting in $p < 0.05$ were considered statistically significant.

RESULTS

Hesperetin protected against 6-OHDA-mediated decrease in cell viability

As expected, treatment of SH-SY5Y cells with 6-OHDA (100 μM) decreased cell viability and promoted LDH release relative to healthy control cells. However, pre-treatment with hesperetin reversed these toxic effects in a dose-dependent manner (Figure 1).

Hesperetin protected against 6-OHDA-mediated apoptosis

Treatment with 6-OHDA led to increased apoptosis relative to control cells (Figure 2 A).

However, pre-treatment with hesperetin reversed this effect (Figure 2 A). Expression of several proteins involved in apoptosis, such as cleaved caspase-3 and -9, was increased after incubation with 6-OHDA, but this effect was attenuated in cells pre-treated with hesperetin (Figure 2 B).

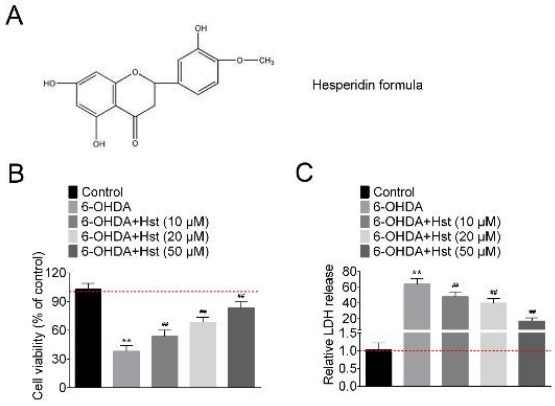

Figure 1: Hesperetin reversed the effects of 6-OHDA on cell viability. (A) Chemical structure of hesperetin (B) Cell viability was measured by MTT assay. (C) LDH release was measured; $^{\#}p < 0.05$; $^{**, \#\#}p < 0.01$

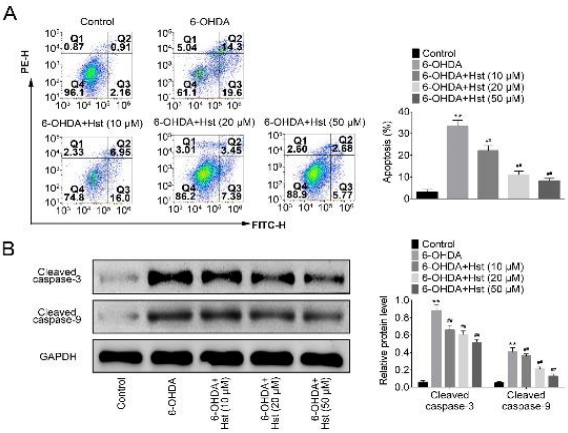

Figure 2: Hesperetin reduced apoptosis of cells treated with 6-OHDA. (A) Cellular apoptosis was detected by flow cytometry. (B) Protein expression was detected by western blots; $^{**, \#\#}p < 0.01$

Hesperetin reversed the effects of 6-OHDA on oxidative stress

The expression of antioxidant enzymes, including GSH and SOD, was decreased by 6-OHDA treatment. However, hesperetin reversed these results (Figure 3 A and B). Similarly, the levels of MDA and ROS were increased after treatment with 6-OHDA but were relatively suppressed after pre-treatment with hesperetin (Figure 3 C and D).

Hesperetin reversed the effects of 6-OHDA on NRF2 pathway

Treatment with 6-OHDA decreased the expression of NRF2, HO-1, GCLC, and GCLM compared to control cells (Figure 4). However, pre-treatment with hesperetin caused over activation of the NRF2 pathway, as seen by relative increases in expression of NRF2, HO-1, GCLC, and GCLM (Figure 4).

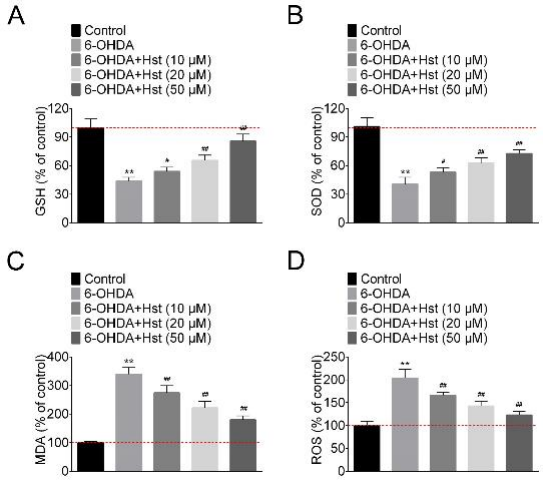

Figure 3: Hesperetin reversed the effects of 6-OHDA on GSH (A), SOD(B), MDA(C) and ROS(D) level. $^{\#}p < 0.05$; $^{**, \#\#}p < 0.01$

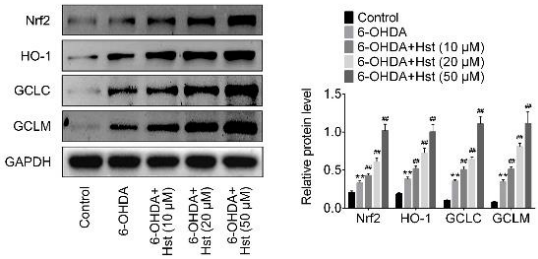

Figure 4: Hesperetin reversed the effects of 6-OHDA on the NRF2 pathway. Western blotting was used to measure protein expression. $^{**, \#\#}p < 0.01$

DISCUSSION

Flavonoids may play a useful role in the prevention and treatment of PKD due to their antioxidant activities [12]. Naringenin, a flavonoid found in grapefruit, has been shown to provide neuroprotection in PKD [13]. In this work, hesperetin, a flavonoid, had neuroprotective effects in a cell model of PKD through its antioxidant and anti-apoptotic effects.

Treatment with 6-OHDA promotes ROS accumulation and apoptosis of dopaminergic cells in rats [14] and is thus commonly used to

create *in vitro* models of PKD [15]. Importantly, SH-SY5Y cells express tyrosine hydroxylase and mimic dopaminergic neurons [16]. Studies have shown that 6-OHDA promotes oxidative stress by uncoupling oxidative phosphorylation and inducing toxicity, similar to native pathological processes in PKD [17]. In this work, SH-SY5Y cells were treated with 6-OHDA to create an *in vitro* model of PKD. The death of dopamine neurons is one of the characteristics of PKD. Therapeutics that counteract the effects of neurotoxins, such as 6-OHDA, to protect neurons may prove valuable for the treatment of PKD [18].

This study showed that pre-treatment with hesperetin reversed 6-OHDA-mediated reductions in cell viability, consistent with a previous report showing that hesperetin protected SH-SY5Y cells against rotenone-induced toxicity [19]. Necrosis and apoptosis are major forms of cell death during PKD [20]. A previous study indicated that LDH is released into the supernatant when necrosis occurs [18]. However, another report revealed that pre-administration of hesperetin decreased neuronal cell apoptosis [21]. Consistent with these prior studies, we found that treatment with hesperetin demonstrated protective effects against 6-OHDA toxicity through inhibition of LDH release and prevention of apoptosis.

PKD has been suggested to be the result of free radical-induced oxidative stress [22]. Under normal conditions, various antioxidant enzymes, including GSH and SOD, detoxify the cell by removing free radicals [23]. The accumulation of MDA and ROS can upset the balance of antioxidant defense systems and result in oxidative stress [2]. Previous studies showed that hesperetin exerts a neuroprotective effect by activating the antioxidant enzyme system and inhibiting oxidative damage [24] and prevents ROS accumulation in a rotenone-induced SH-SY5Y cell model [19]. Consistently, this study found that hesperetin had antioxidant effects in cells treated with 6-OHDA treatment, primarily through increasing the expression of antioxidant enzymes (GSH and SOD) and reducing the relative concentration of markers of oxidative stress (MDA and ROS).

Several signaling pathways play important roles in the antioxidant response to PKD [25]. NRF2 is a key regulator of cytoprotective genes, such as *HO-1*, *GCLC*, and *GCLM,* and plays a central role in managing oxidative stress during PKD [13]. Activation of the NRF2 pathway, as evidenced by the increased expression of NRF2, HO-1, GCLC, and GCLM, leads to neuroprotection of SH-SY5Y cells treated with 6-OHDA [13]. A previous study showed that hesperetin exerts neuroprotective effects by activating the NRF2 pathway [26]. Similarly, we found that hesperetin upregulated NRF2, HO-1, GCLC, and GCLM, thus activating the NRF2 pathway and reducing oxidative stress. However, further investigations in animal models of disease will be required to more completely reveal the neuroprotective role of hesperetin in the context of PKD.

CONCLUSION

Hesperetin protects against apoptosis and oxidative stress in a 6-OHDA-induced SH-SY5Y cell model of PKD. These antioxidant effects were mediated through activation of the NRF2 pathway. These findings indicate that hesperetin may have a neuroprotective role in the management of PKD.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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