Punicalagin Exerts Beneficial Functions in 6-Hydroxydopamine-Treated SH-SY5Y Cells by Attenuating Mitochondrial Dysfunction and Inflammatory Responses

Background: Parkinson’s disease (PD) is a common age-related neurodegenerative disorder, but effective therapeutic agents for PD remain largely limited.

Material/Methods: In the present study, we evaluated the beneficial effects and underlying mechanisms of punicalagin (PN) in human neuroblastoma SH-SY5Y cells treated with 6-hydroxydopamine (6-OHDA) to mimic PD in vitro. Cell viability was monitored by MTT assay and LDH release assay. Cell apoptosis was assayed by Annexin V-FITC/PI double-staining. Intracellular ROS production was assessed by DCFH-DA staining. The expression levels of protein and mRNA were determined by Western blotting and qRT-PCR analysis, respectively.

Results: The results showed that pretreatment of SH-SY5Y cells with PN (50, 100, and 200 µM) prior to exposure to 200 µM 6-OHDA for 2 h resulted in increased cell viability and decreased cell apoptosis. PN also inhibited excessive oxidative stress in 6-OHDA-treated SH-SY5Y cells. Moreover, PN treatment effectively restored mitochondrial function and enhanced phosphorylation of AMPK. Furthermore, PN blocked 6-OHDA-induced NF-κB activation and IL-1β expression.

Conclusions: Our study shows that PN exhibited neuroprotective effects on the 6-OHDA-treated SH-SY5Y cells, thus providing a potential theoretical insight for the clinical application of PN against PD.

MeSH Keywords: AMP-Activated Protein Kinases • NF-kappa B • Oxidative Stress • Parkinson Disease

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Background

Parkinson’s disease (PD), a neurodegenerative, progressive disorder first reported by James Parkinson in 1817 [1], affects about 1% of the population over the age of 50 years [2]. AD is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta, along with the abnormal aggregation of α-synuclein in Lewy bodies [3]. PD severely affects a patient’s physical and mental condition and imposes a substantial economic burden on society. Therefore, novel effective drugs with limited adverse effects and other forms of therapeutic approaches for PD are required.

Drug development for PD remains a major challenge. In recent years, considerable attention has been paid to the beneficial effects of natural products in neurodegenerative diseases, including PD [4]. Punica granatum (pomegranate) is an antioxidant-rich fruit. Punicalagin (2,3-hexahydroxydiphenoyl-gallagyl-D-glucose; PN; Figure 1) is the major antioxidant polyphenol ingredient in pomegranate, and it is beneficial for overall good health. It is well established that PN possesses many physiological activities, including strong anti-oxidative and anti-inflammatory properties. For example, PN has been reported to exert inhibitory effects on lipopolysaccharide-induced neuroinflammation and oxidative stress in cultured astrocytes and microglial BV-2 cells [5]. However, there are limited reports on the protective activities of PN in PD.

6-hydroxydopamine (6-OHDA) is a synthetic organic compound widely used to generate an experimental cellular model of PD in vitro [6]. In the present study, we used 6-OHDA to induce cytotoxicity in human neuroblastoma SH-SYSY cells to investigate the potential protective role of PN in PD, as well as the related underlying mechanisms.

Material and Methods

Cell culture and drug treatment

Human neuroblastoma cell line SH-SYSY cells, obtained from American Type Culture Collection (ATCC; Manassas, VA, USA), were plated onto culture plates coated with poly-d-lysine and maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin-streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. SH-SYSY cells were treated with 200 µM 6-OHDA (dissolved in ascorbic acid; Sigma, St. Louis, MO, USA) equipped with Cell Quest software. In brief, after various treatments, the cells were incubated at 37°C. SH-SYSY cells were collected, washed, and resuspended in Annexin V-FITC and stained with 5 µl PI at room temperature for 15 min in the dark. The apoptosis rates were detected by FACScan flow cytometer (BD Biosciences, Franklin lakes, NJ, USA) equipped with Cell Quest software.

Measurement of intracellular ROS accumulation

Intracellular ROS production was assessed with 2’, 7’-dichlorofluorescein diacetate (DCFH-DA) fluorescent probe (Sigma). In brief, after various treatments, the cells were incubated with 20 µl of MTT solution (5 mg/ml; Sigma) at 37°C. After another 4 h, the supernatants were replaced with DMSO to dissolve the formazan crystals, and the absorbance was measured with a microplate reader (Dynex, Chantilly, VA, USA) at a wavelength of 570 nm.

Cell viability analysis

Cell viability was monitored by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After the aforementioned treatments, SH-SYSY cells were treated with 20 µl of MTT solution (5 mg/ml; Sigma) at 37°C. After another 4 h, the supernatants were replaced with DMSO to dissolve the formazan crystals, and the absorbance was measured with a microplate reader. Released LDH was normalized to total LDH.

Cell apoptosis analysis

Cell apoptosis was assayed by an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis kit (MultiSciences Biotech, Co., Ltd., Suzhou, China) [7]. Briefly, after treatments, SH-SYSY cells were collected, washed, and resuspended in the binding buffer. Then, the cells were incubated with 5 µl Annexin V-FITC and stained with 5 µl PI at room temperature for 15 min in the dark. The apoptosis rates were detected by FACScan flow cytometer (BD Biosciences, Franklin lakes, NJ, USA) equipped with Cell Quest software.

Figure 1. The chemical structure of punicalagin (PN).
with DCFH-DA (10 μM) in the dark at 37°C for 30 min. Then, the cells were washed 3 times with PBS and the fluorescence was monitored by flow cytometry.

**Measurement of superoxide dismutase (SOD) activity**

Superoxide dismutase (SOD) activity was measured using a commercial SOD assay kit (Jiancheng). After treatments, the cells were homogenized and lysed on ice. Then, lysates were centrifuged and the supernatant was collected to analyze the SOD activity. The luminescence was measured using a microplate reader.

**Measurement of intracellular ATP level**

The ATP levels in the cells were detected using a commercial ATP assay kit (Beyotime). In brief, after treatments, the cells were incubated with the assay buffer with shaking for 15 min at room temperature. Then, the chemiluminescence of the samples was detected using a microplate reader.
Measurement of mitochondrial membrane potential (MMP)

The MMP was measured using the dye 5,5'6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1; Cayman Chemical Co., Ann Arbor, MI, USA). At high membrane potentials, JC-1 enters the mitochondria and forms aggregates that have a red fluorescence, whereas JC-1 shows as a green fluorescence at a low potential. Briefly, after the aforementioned treatments, the cells were washed with PBS and incubated with 0.5 ml JC-1-fluorescent dye for 30 min at 37°C in the dark. The fluorescence of green monomer and red aggregates were quantified by flow cytometry. Results are expressed as the ratio of the red/green fluorescence.

Western blotting

Cellular proteins were prepared using RIPA lysis buffer (Beyotime). For detection of cytochrome c, the mitochondrial and cytosolic fractions were prepared using the Mitochondria/Cytosol Fractionation kit (Abcam, Cambridge, MA, USA). For detection of NF-κB p65, the nuclear and cytosolic fractions were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Rockford, IL, USA). The protein concentrations were assessed using a BCA protein assay kit (Beyotime) [8]. Lysates containing equivalent amounts of protein were loaded on SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Then, the membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with HRP-conjugated secondary antibodies for 2 h at room temperature. Protein bands were visualized using the enhanced chemiluminescence detection system (Amersham, Little Chalfont, UK). GAPDH, COX IV, or Lamin B was considered as a loading control.

Quantitative reverse transcription-PCR (qRT-PCR)

The mRNA levels of IL-1β were measured by quantitative reverse transcription-PCR (qRT-PCR). After the aforementioned treatments, total RNA was isolated from the cells using the Trizol reagent (Invitrogen). First-strand cDNA was synthesized using the reverse transcription kit (Takara, Tokyo, Japan). qPCR was performed on the LightCycler 480 System II (Roche Diagnostics, Rotkreuz, Switzerland) using the fluorescent dye SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). The data were calculated using the 2-ΔΔCt method with GAPDH serving as an internal control [9]. The sequences of primers used here were shown as follows: IL-1β, forward primer: 5'-GAGCTCGCCAGTGAAATGATG-3', reverse primer: 5'-AGTGGTGGTCGGAGATTCGT-3'; GAPDH, forward primer: 5'-ACAGTCAGCCGCATCTTCTT-3', reverse primer: 5'-GACAAGCTTCCCGTTCTCAG-3'.

Statistical analysis

All results are expressed by the mean ± standard deviation (SD) and the data were from at least 3 independent experiments performed in triplicate. One-way ANOVA followed by Tukey's multiple comparison were performed using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). The difference was considered as significant when the P-value was less than 0.05.

Results

PN attenuates 6-OHDA-induced cytotoxicity in SH-SY5Y cells

First, as indicated by MTT assay, PN alone had no obvious effect on SH-SY5Y cell viability (Figure 2A). Then, the effects of

![Figure 3. PN reduces 6-OHDA-induced oxidative stress in SH-SY5Y cells. (A) Intracellular ROS accumulation was detected through DCFH-DA staining. (B) Intracellular SOD activity was estimated using a commercial kit. *P<0.05 vs. 6-OHDA-untreated cells; #P<0.05 vs. PN-untreated cells.](image-url)
Figure 4. PN attenuates 6-OHDA-induced mitochondrial dysfunction in SH-SY5Y cells. (A) The disruption of MMP was determined through JC-1 staining. (B) Intracellular ATP level was estimated using a commercial kit. (C) Western blot analysis was performed to detect the protein levels of cytochrome c in the cytosolic and mitochondrial fractions. (D) Western blot analysis was performed to detect the protein levels of cleaved caspase-3. * P<0.05 vs. 6-OHDA-untreated cells; # P<0.05 vs. PN-untreated cells.
PN on the 6-OHDA-treated SH-SY5Y cells were determined, and we found that the average viability of the cells treated with 6-OHDA alone was reduced to approximately 40%, and PN pretreatment exerted protective effects against 6-OHDA-induced injury (Figure 2B). The protective role of PN in 6-OHDA-injured SH-SY5Y cells was further confirmed by LDH release assay. As expected, the LDH release was significantly eliminated by PN pretreatment (Figure 2C). SH-SY5Y cells were further stained with apoptosis marker Annexin V/PI and analyzed with flow cytometry, and the results demonstrated that PN dose-dependently rescued SH-SY5Y cells from 6-OHDA-induced apoptosis (Figure 2D).

**PN reduces 6-OHDA-induced oxidative stress in SH-SY5Y cells**

ROS accumulation and the induced oxidative stress play a critical role in cell apoptosis [10]. The level of intracellular ROS was examined by DCFH-DA staining. The results showed that SH-SY5Y cells treated with 6-OHDA showed a significant increase of intracellular ROS, and this increase was significantly restored by PN pretreatment (Figure 3A). Administration of PN also dose-dependently enhanced the SOD activity in 6-OHDA-treated SH-SY5Y cells (Figure 3B).

**PN attenuates 6-OHDA-induced mitochondrial dysfunction in SH-SY5Y cells**

To further assess the effects of PN on mitochondrial injury induced by 6-OHDA, we analyzed the MMP of treated cells by JC-1 staining. The results showed that PN remarkably restored the 6-OHDA-induced disruption of MMP (Figure 4A). Consistently, we also observed that PN pretreatment increased the intracellular ATP levels in the 6-OHDA-treated cells (Figure 4B). Furthermore, as shown in Figure 4C, 6-OHDA treatment resulted in an increase of cytosolic cytochrome c, whereas PN significantly reduced the cytochrome c release in a dose-dependent manner. The mitochondria-mediated apoptosis involves the activation of caspase cascade. Here, we also observed that PN dose-dependently attenuated the increased expression levels of cleaved caspase-3 induced by 6-OHDA exposure (Figure 4D).

**PN rescued 6-OHDA-induced phosphorylation of AMPK in SH-SY5Y cells**

As demonstrated in Figure 5A, we found that 6-OHDA exposure could promote phosphorylation of AMPK. However, intriguingly, pretreatment of PN enhanced the phosphorylation levels of AMPK. We then chose compound c as the AMPK inhibitor to analyze whether AMPK inhibition could block the protective role of PN in 6-OHDA-induced cell apoptosis. The p-AMPK level was significantly reduced due to the application of AMPK inhibitor to 6-OHDA-untreated cells. Interestingly, pretreatment of PN enhanced the phosphorylation of AMPK (Figure 5B).
Figure 6. PN suppresses NF-κB activation and inhibits IL-1β expression in 6-OHDA-treated SH-SY5Y cells. (A) Western blot analysis was performed to detect the protein levels of IκBα. (B) Western blot analysis was performed to detect the protein levels of p65 in the cytosolic and nuclear fractions. (C) qRT-PCR was performed to detect the mRNA levels of IL-1β. * P<0.05 vs. 6-OHDA-untreated cells; # P<0.05 vs. PN-untreated cells.
of compound c (data not shown), and 5 μM of compound c remarkably increased the apoptotic rate of SH-SYSY cells pretreated with PN before 6-OHDA exposure (Figure 5B).

**Discussion**

Many studies have demonstrated that natural products derived from fruits and vegetables have neuroprotective effects. In the present study, we established a sensitive PD model using 6-OHDA on SH-SYSY cell line due to its neuronal background and neuron-like properties [11], and observed that 6-OHDA exposure led to impaired viability and increased apoptosis of SH-SYSY cells. These effects were significantly counteracted by pretreatment of PN. These experimental results clearly illustrate the neuroprotective effects of PN on 6-OHDA-injured SH-SYSY cells.

ROS increases with age, and oxidative stress caused by excessive ROS is closely related to the pathogenesis of PD [12]. The mitochondrion is a critical organelle for cellular metabolism, and the impairment of mitochondrial function is also linked to aging and neurodegeneration [13]. 6-OHDA can produce oxidative stress, thereby inducing a ROS-related collapse in MMP [14,15]. Here, we found that PN eliminated ROS production, stabilized MMP, and therefore inhibited cytochrome c release in 6-OHDA-treated SH-SYSY cells. Cytochrome c release further induces the activation of the caspase cascade, thus eventually leading to apoptosis [16]. In the present study, the cleavage of caspase-3 was also significantly suppressed by PN pretreatment.

**Conclusions**

Our findings provided direct experimental evidence that PN exerts its neuroprotective effects against 6-OHDA-induced cytotoxicity in SH-SYSY cells through attenuating mitochondrial dysfunction and inflammatory responses. Although the in vivo effects require further exploration, the present study provides support for the potential role of PN in preventing neuronal cell death in the process of PD.

**Conflict of interest**

None.

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