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Original Article

Protective effect of nobiletin against apoptosis induced by 6-hydroxydoamine in human neuroblastoma cells

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Abstract

Introduction: Parkinson's disease (PD) is the second most common neurodegenerative disorder characterized by the damage of dopaminergic neurons of substantia nigra. Despite considerable research, therapeutic approaches aimed at the prevention and long-term treatment of PD have not been quite successful. Therefore, there is a tendency for the identification of novel medical intervention derived from natural substances. Nobiletin, an important citrus flavonoid commonly present in sweet and bitter orange peel, has been suggested to act as a neuroprotective agent in animal models of PD. This study was aimed to assess the potentials of nobiletin in preventing neuronal death and caspase-3 in SH-SY5Y cells.

Methods: SH-SY5Y cells were grown in DMEM/F12 media supplemented with 10% fetal bovine serum. The 6-hydroxydopamine (6-OHDA) with or without nobiletin was added to cells. After 24h, the cells were examined for morphological changes under a light microscope and viability by MTT assay. The protective doses of nobiletin was chosen through a pilot study and accordingly the doses 50 and 250µM were selected for further assessments. Western blot assays were done to examine the effect of 6-OHDA with/without nobiletin on cleaved (active) caspase-3.

Results: Our results showed that nobiletin is effective in attenuating the effect of 6-OHDA on cell viability by the MTT assay. Nobiletin also reduced the cleavage of caspase-3 induced by 6-OHDA.

Conclusion: These results suggest that nobiletin has protective effects against dopaminergic neural toxicity and its anti-apoptotic effect is involved, at least in part, in such protection.

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Keywords:
Nobiletin;
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Introduction

Nobiletin is a polymethoxylated flavone present in citrus fruits (Sasaki and Yoshizaki, 2002). Nobiletin is reported to exert some favorable effects, like anti-oxidative, anti-inflammatory and immunomodulatory (Huang et al., 2016). Several lines of evidence have also shown its neuroprotective effects in animal models of Alzheimer's disease (AD) (Onozuka et al., 2008) and Parkinson's disease (PD) (Yabuki et al.,

2014; Jeong et al., 2015; Braidy et al., 2017).

PD, the second most prevalent neurodegenerative disease (Lees et al., 2009), is characterized with dopaminergic neuronal loss in substantia nigra and intracytoplasmic substances named Lewy bodies (Forno, 1996). Levodopa is a well-known treatment of PD, but its long term use leads to the side effects including farther oxidative stress (Dorszewska et al., 2014).

Apoptosis has been suggested as a plausible mechanism in PD pathogenesis (Khan et al., 2015). Considerable *in vitro* studies both in non-neuronal and neuronal cells demonstrated caspases are the effectors of apoptosis (Cohen, 1997). In neurons, increasing evidence indicated that caspase-3 plays an important role in the executive stage of apoptosis (Chaudhry and Ahmed, 2013). Activated caspase-3 has been shown in the substantia nigra of PD patients and therefore caspase-3 has been implicated in the pathogenesis of PD (Hartmann et al., 2000). In animal and cellular models of PD, the neurotoxins frequently used to induce experimental PD, such as 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenylpyridinium (MPP^+) have been reported to activate caspase-3 to exert their pro-apoptotic effects (Dodel et al., 1999; Lotharius et al., 1999). However, gene disruption of caspase-3 has been reported to prevent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine- (MPTP)-induced PD in mice (Yamada et al., 2010).

The SH-SY5Y neuroblastoma cell line is widely used to generate a cellular model of PD (Xie et al., 2010) since they share many functional and biochemical properties with mature dopaminergic neural cells. The 6-OHDA, as an analog of dopamine, has a hydroxyl group which specifically damages dopaminergic neurons making that toxic for dopaminergic neural cells (Ferger et al., 2001). Exposure to 6-OHDA leads to apoptosis in dopaminergic cells of substantia nigra (He et al., 2000) and SH-SY5Y cell line (Ikeda et al., 2008; Amiri et al., 2016). In the present study we aimed to examine the protective effect of nobiletin against 6-OHDA toxicity in human SH-SY5Y cell line.

Materials and methods

Materials

Human neuroblastoma cell line (SH-SY5Y) was purchased from Pasteur institute of Iran. Cell culture materials including Dulbecco's modified Eagle medium (DMEM/F12, 32500-035), fetal bovine serum (FBS, 10270-106), L-glutamine (10270106) and penicillin-streptomycin (15140-122), were purchased from Gibco life technologies. Nobiletin (N1538), 6-OHDA (H4381) and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, M5655) were purchased from Sigma-Aldrich. Western blot antibodies including caspase-3 antibody (9665), beta-actin antibody (4970) and secondary HRP-conjugated antibody (7074) were purchased from Cell Signaling Technology. Amersham ECL select (RPN2235) reagent kit was purchased from GE health care. Bovine serum albumin (BSA, 1120180100) and polyvinylidene difluoride (PVDF) membrane (IPVH00010) was purchased from Millipore. Other reagents were obtained from usual commercial sources.

Cell culture

SH-SY5Y cells were cultured in DMEM/ F12 supplemented with 10% heat-inactivated FBS, 2mM L-glutamine, 50U/ml penicillin and 50µg/ml streptomycin. The cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The cells were used in the exponential growth phase and below passage 10 in all experiments. For cell viability studies, the cells were plated in 96 well and for western blotting studies, 6-well plates were used.

Cell treatment

The 6-OHDA, dissolved in sterile distilled water which contained 0.1% ascorbic acid as a stock solution (6mM), was added to cell culture medium to achieve the required concentration (50µM (Amiri et al., 2016; Moosavi et al., 2018; Sookhaklari et al., 2019). Nobiletin was stored at -20°C as a stock solution of 100mM in dimethyl sulfoxide (DMSO). The 6-OHDA and nobiletin were diluted in cell culture media immediately before use. To acquire the protective doses of nobiletin, a pilot test was done and the doses 1, 5, 10, 20, 50, 75, 100, 150, 200 and 250µM (Cui et al., 2010; Nemoto et al., 2013) were assessed. According to the results of this preliminary study, nobiletin at doses 50 and 250µM (chosen as the minimum and the maximum protective doses) was used for the following experiments.

Six cell groups were prepared and the control group was exposed to vehicles of the drugs. Cells in the 6-

OHDA group were exposed to 50μM 6-OHDA for 24 hours. Cells in the 6-OHDA+nobiletin group were exposed to 50μM 6-OHDA and nobiletin at the dose of 50 or 250μM for 24 hours. Cells in the nobiletin groups were treated with nobiletin at dose 50 or 250μM for 24 hours to assess any possible interaction effects.

Assessment of cell viability and morphological changes

Cell viability was detected using colorimetric MTT assay. SH-SY5Y cells were seeded in 96-well plates at a density of 1×10^4 cells/well and grown for 24h. Afterwards, the cells were incubated with 6-OHDA and/or nobiletin for 24 hours. The cells incubated in culture media containing an equivalent concentration of DMSO (less than 0.1%) were considered as the control cells. Thereafter, the cells were incubated in fresh MTT solution/cell culture media (final concentration 0.5mg/ml) for 4h at 37°C. Then, the medium was removed and DMSO (100μl/well) was added to dissolve the formazan crystals. Following 15min of homogenization, the cell viability was determined by absorbance of samples at 570nm using a microplate reader (Synergy HT, Biotek®). Morphological changes were observed under a light microscope (Nikon, ECLIPSE Ts2).

Western blot assays

SH-SY5Y cells were washed with phosphate-buffered saline and lysed with ice-cold RIPA (Radioimmunoprecipitation assay) buffer containing 50mM Tris-HCl pH=8.0, 150mM NaCl, 1% Triton X-100, 0.5% Na-Deoxycholate and 0.1% SDS (sodium dodecyl sulfate) supplemented with protease and phosphatase inhibitor cocktail. Following incubation for 30min at 4°C, the samples were centrifuged at 13,000 rpm for 30min. The protein levels were determined using Lowry method (Lowry et al., 1951). Briefly, equal amounts of protein were separated by electrophoresis in 10% SDS polyacrylamide gels and transferred onto PVDF membranes. The membranes were incubated with 5% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1h to block nonspecific binding. Afterwards, the membranes were incubated overnight at 4°C with primary antibodies against caspase-3 (1/3000) and β-actin (1/4000). After washing with TBST, the membranes were incubated for 1 hour at room temperature with secondary horseradish peroxidase-conjugated antibodies (anti-rabbit IgG, 1/10000). The membranes were washed again with TBST and the immunolabeling developed using ECL select kit. The protein levels were quantified by densitometry using Image J software.

Statistical analysis

The data are expressed as the mean±SEM of 3 independent experiments. One-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test was performed for multiple group comparisons using GraphPad Prism software, version 6. A value of $P<0.05$ was considered statistically significant.

Results

The effects of 6-OHDA and/or nobiletin on the viability of SH-SY5Y cells

According to MTT assay results, 6-OHDA decreased the viability of SH-SY5Y cells (Fig. 1). In order to obtain the protective doses of nobiletin, a pilot study was done and the doses 1, 5, 10, 20, 50, 75, 100, 150, 200 and 250μM were exposed to cells. The analysis by one-way ANOVA revealed a significant difference ($F(11, 24)= 5.205$, $P=0.0004$). Tukey's post hoc test indicated that nobiletin exert a protective effect at doses ≥ 50μM (Fig. 1). Therefore, the doses 50 and 250μM were selected for further assessment. Figure 2 compares cell viability when nobiletin at dose 50 or 250μM was added. One-way ANOVA indicated a difference between groups ($F(5,12)= 8.346$, $P=0.0013$). Tukey's post hoc test revealed that when nobiletin was added to 6-OHDA treated cells, it attenuated the effect of 6-OHDA on cell viability while nobiletin (50 or 250μM) alone did not affect cell viability comparing to control group (Fig. 2).

The effects of 6-OHDA and/or nobiletin on morphology of SH-SY5Y cells

As depicted in Figure 3, following 6-OHDA treatment, cell shrinkage occurs, the cell loses its particular neuronal shape and its size decreased. Treatment with nobiletine at doses 50 and 250μM attenuates these changes.

Nobiletin reversed 6-OHDA- induced caspase-3 cleavage

To assess the level of cleaved caspase-3 western blot technique was used. Cleaved caspase-3

Fig. 1

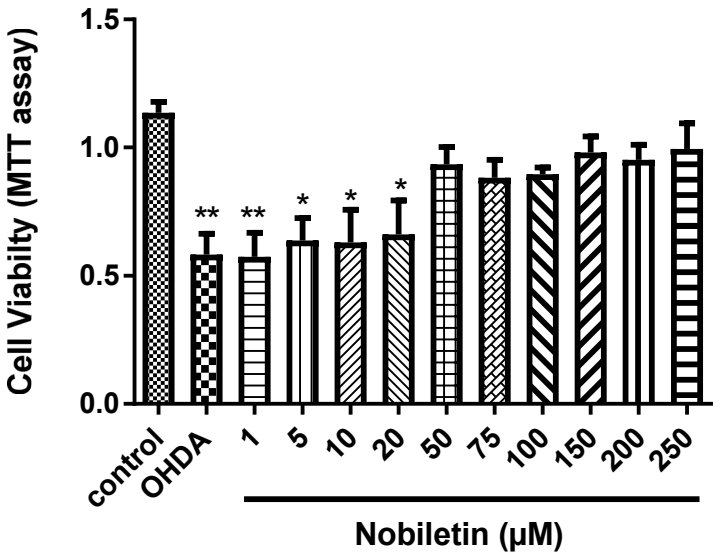

Fig.1. Effect of nobiletin at doses 1, 5, 10, 20, 50, 75, 100, 150, 200 and 250μM on cell viability changes of 6-OHDA treated SH-SY5Y cells. Cell viability was assessed through MTT assay and data are shown as mean±SEM of three independent experiments. $^{*}P<0.05$ and $^{**}P<0.01$ show the difference versus control cells.

Fig. 2

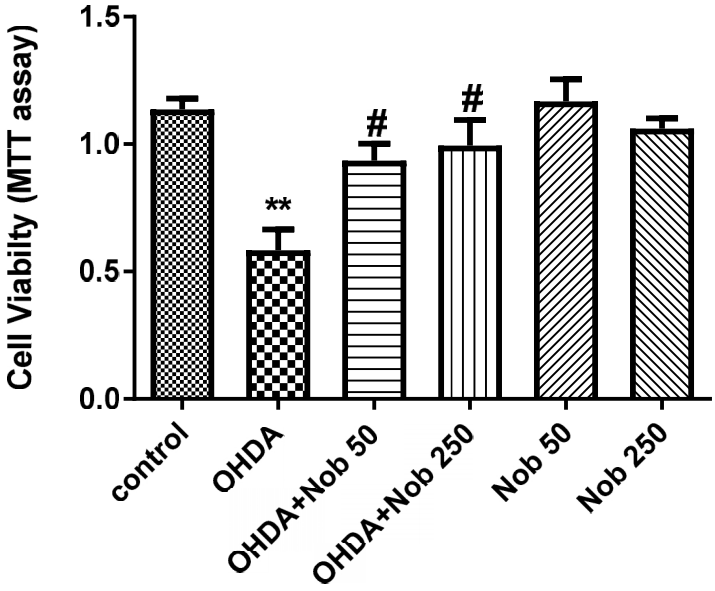

Fig.2. Effect of selected doses of nobiletin (50 and 250μM) on 6-OHDA-treated SH-SY5Y cells. Cell viability was determined via MTT assay and data shown are mean±SEM of three independent experiments. $^{**}P<0.01$ represents the difference versus control cells while $^{\#}P<0.05$ shows the difference versus 6-OHDA treated cells and nobiletin co-treated ones.

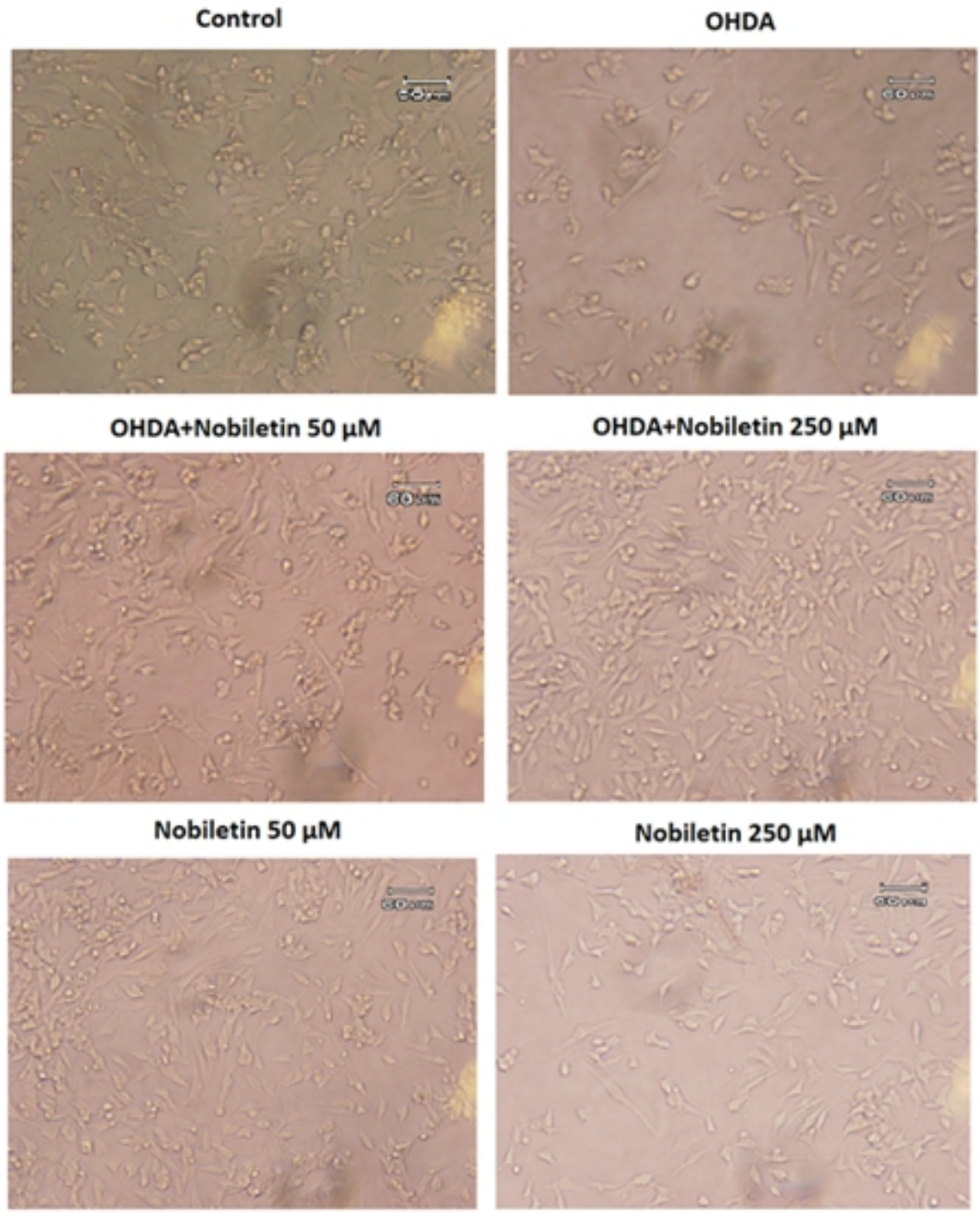

Fig.3. Effect of nobiletin at doses 50 and 250µM on morphological changes induced by 6-OHDA. Microscopic images were taken 24h after treatment. Following 6-OHDA treatment, cell shrinkage occurs, the cell loses its particular neuronal shape and its size decreased. Treatment with nobiletine at doses 50 and 250µM attenuates these changes.

appeared as a band at 19kDa (Fig. 2A). One-way ANOVA revealed a significant difference between groups (F (3, 8)=18.03, *P*=0.0006). Tukey's post hoc test, indicated that 6-OHDA exposure leads to a significant increase in caspase-3 cleavage (*P*<0.001), while nobiletin co-treatment reduced the level of cleaved caspase-3 at doses 50 (*P*< 0.05) and 250µM (*P*< 0.01).

Discussion

Since there is a lack of efficient therapies for PD, the approach of neuroprotection attributing to the

Fig. 4

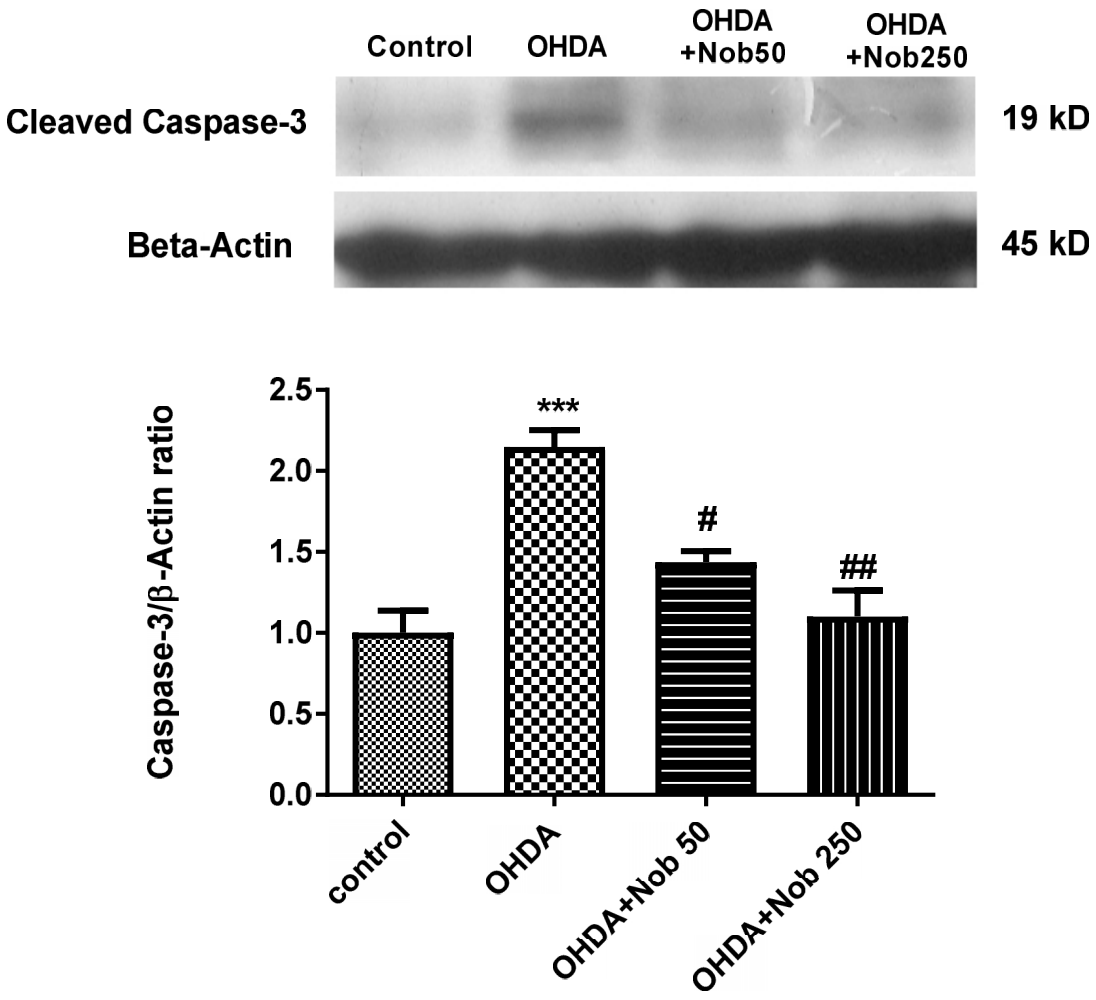

Fig.4. Effect of nobiletin at doses 50 and 250μM on 6-OHDA-induced caspase-3 cleavage in SH-SY5Y cells. Cells of different groups were analyzed by western blot technique to determine the level of cleaved caspase-3. The graph represents the densitometry results which are shown as mean±SEM of three independent experiments. $^{***}P<0.001$ shows the difference versus control cells while $^{\#}P<0.05$ and $^{\#\#}P<0.01$ indicate the difference versus 6-OHDA treated cells.

prevention of dopaminergic cell death, seems to be a hopeful concept. In this field, natural polyphenols such as nobiletin have been much considered during recent decade (Braidy et al., 2017). In the present study, the human neuroblastoma cell line exposed to 6-OHDA was used as an *in vitro* model of PD. The results indicated that: 1) the incubation of cells with 6-OHDA for 24h decreased cell viability by about 50%. Using the MTT assay, it was also revealed that nobiletin co-treatment decreased the cell loss induced by 6-OHDA exposure; 2) there was a significant increase in the level of cleaved caspase-3 in 6-OHDA treated neuroblastoma cells while co-treatment with nobiletin at doses 50 and 250μM reversed the increase in the cleaved caspase-3 level.

The selective loss of dopaminergic neural cells of the substantia nigra is assumed as the direct cause of PD neurodegeneration and apoptosis has also been proposed to take part in this process (Anglade et al., 1997). SH-SY5Y cells, a sub line of human neuroblastoma cells (Xing et al., 2005), own some comparable characters with dopaminergic cells (Song et al., 2012) and are considered as a suitable cellular model of PD. The 6-OHDA, as a hydroxylated analogue of dopamine, is one of the common agents used to produce PD-like neural cell degeneration both *in vivo* and *in vitro* (Blum et al., 2001). In the present study, treatment with 50μM 6-OHDA resulted in mitochondrial activity alteration, as verified by MTT assay. The 6-OHDA, move in the cells through dopaminetransporter (Ljungdahl et al., 1971) and generates intracellular free radicals which triggers the activation of cell death pathways (Hwang, 2013). Similarly, it was suggested that the impairment of

mitochondrial function and the resultant oxidative stress play roles in PD pathogenesis (Gu et al., 1998). When SH-SY5Y cells were treated with nobiletin, it reversed 6-OHDA effect on cell viability at doses equal or higher than 50µM. This result might be due to the antioxidant properties of nobiletin since evidence indicated that nobiletin exerts antioxidant effects both *in vivo* and *in vitro* (Zhang et al., 2016; Liu and Wu, 2018). Consistent with the findings of the present study, in animal models of PD, nobiletin is found to exert some protective effects. In MPTP triggered mice PD, nobiletin increases dopamine release in striatum and hippocampal CA1. It also restores the attenuated activity of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and cAMP-regulated phosphoprotein-32 (DARPP-32), restores the decreased activity of tyrosine hydroxylase (TH) and rescues MPTP-induced cognitive and motor dysfunctions (Yabuki et al., 2014). In the same way, in MPP^{+}-triggered rat PD, nobiletin not only protected dopaminergic neurons of substantia nigra, but also inhibits microglial activation and restores the expression of neurotrophic factor derived from the glial cells (Jeong et al., 2015).

Several studies identified the role of the mitochondrial-caspase cascade following 6-OHDA exposure, which triggers the activation of the main effector caspases-3 (Woodgate et al., 1999; Amiri et al., 2016). In agreement, the cleavage of caspase-3 was detected to be considerably increased in 6-OHDA treated cells in the present study. Likewise, human postmortem studies suggested that, dopaminergic neurons die through apoptosis in PD (Anglade et al., 1997; Kingsbury et al., 1998) and the increase of caspase-3 immunoreactivity were shown in those neurons (Tatton, 2000). A number of investigations have shown the anti-apoptotic effect of nobiletin as a mechanism for its protective effect. For instance, nobiletin inhibited the apoptotic process in ischemic/reperfusion-exposed Kupffer cells after liver transplantation (Wu et al., 2017). Additionally, in pressure overload-induced myocardial damage (Zhang et al., 2017; Mao et al., 2019) and injured PC12 cells (Li et al., 2018), nobiletin reduced apoptosis. Based on previous investigations, in the present study, we hypothesized that nobiletin might exert anti-apoptotic effect in 6-OHDA exposed SH-SY5Y cells. Accordingly, our study showed that nobiletin reversed caspase-3 cleavage indicating its anti-apoptotic effect. A recent study suggested the involvement of the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway in anti-apoptotic effect of nobiletin (Mao et al., 2019). Activated Akt might exert an anti-apoptotic effect via the regulation of Bax and Bcl-2 which control caspase-3 activation (Salakou et al., 2007). Although Bcl-2 is anti-apoptotic, Bax is regarded as a pro-apoptotic factor and their relative expression determines the cell viability (Howard et al., 2002). Akt was shown to phosphorylate Bad at Ser136 which inactivates its activity and helps to maintain Bcl-2 function (Datta et al., 1997).

Conclusion

In conclusion, the present study showed that nobiletin protects against the lethal effect of 6-OHDA and subsequent apoptosis in a cellular model of PD. To our knowledge this is the first study assessing the effect of nobiletin on human neuroblastoma cells as an *in vitro* model of PD. Since recently nobiletin was suggested to protect against PD an animal models (Yabuki et al., 2014; Jeong et al., 2015), these *in vitro* findings are worthy in elucidating the mechanism of the protective effects of this flavonoid in PD.

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interests.

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