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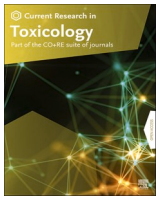

Protective effects of amphetamine and methylphenidate against dopaminergic neurotoxicants in SH-SY5Y cells

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ABSTRACT

Full treatment of the second most common neurodegenerative disorder, Parkinson's disease (PD), is still considered an unmet need. As the psychostimulants, amphetamine (AMPH) and methylphenidate (MPH), were shown to be neuroprotective against stroke and other neuronal injury diseases, this study aimed to evaluate their neuroprotective potential against two dopaminergic neurotoxicants, 6-hydroxydopamine (6-OHDA) and paraquat (PQ), in differentiated human dopaminergic SH-SY5Y cells.

Neither cytotoxicity nor mitochondrial membrane potential changes were seen following a 24-hour exposure to either therapeutic concentration of AMPH or MPH (0.001–10 μM). On the other hand, a 24-hour exposure to 6-OHDA (31.25–500 μM) or PQ (100–5000 μM) induced concentration-dependent mitochondrial dysfunction, assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, and lysosomal damage, evaluated by the neutral red uptake assay. The lethal concentrations 25 and 50 retrieved from the concentration-toxicity curves in the MTT assay were 99.9 μM and 133.6 μM for 6-OHDA, or 422 μM and 585.8 μM for PQ. Both toxicants caused mitochondrial membrane potential depolarization, but only 6-OHDA increased reactive oxygen species (ROS). Most importantly, PQ-induced toxicity was partially prevented by 1 μM of AMPH or MPH. Nonetheless, neither AMPH nor MPH could prevent 6-OHDA toxicity in this experimental model.

According to these findings, AMPH and MPH may provide some neuroprotection against PQ-induced neurotoxicity, but further investigation is required to determine the exact mechanism underlying this protection.

1. Introduction

Parkinson's disease (PD) is the second leading age-related neurodegenerative disorder, with a prevalence of 8.5 million and an incidence of more than 8.5 million in 2019 (Ou et al., 2021). PD is characterized by the degeneration of dopaminergic neurons with subsequent depigmentation of the substantia nigra pars compacta (SNpc) (Vázquez-Vélez & Zoghbi, 2021; Zesiewicz, 2019) and the presence of proteinaceous aggregates in neurons, known as Lewy bodies. The resulting decrease in dopamine (DA) release in the striatum and modification of synaptic function eventually lead to neuronal degeneration (Balestrino & Schapira, 2020; Vázquez-Vélez & Zoghbi, 2021).

Presently, there are no disease-modifying agents available for PD (Zahoor et al., 2018), revealing the unmet need for new drugs as well as models to reproduce the disorder. Neurotoxicants remain the most common tools to induce selective neuronal death in both *in vitro* and in vivo systems to mimic PD features (Bové et al., 2005) or to study putative neuroprotective agents (Kitamura et al., 2002).

Abbreviations: 6-OHDA, 6-hydroxydopamine; AMPH, Amphetamine; CCCP-1, Carbonyl cyanide m-chlorophenyl hydrazone-1; DAT, Dopamine transporter; DCFH-DA, 2′,7′-Dichlorofluorescein diacetate; FBS, Foetal bovine serum; HBSS, Hanks' balanced salt solution; JC-1, Tetraethylbenzimidazolylcarbocyanine iodide; LC, Lethal concentration; MPH, Methylphenidate; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NR, Neutral red; PBS, Phosphate-buffered saline; PQ, Paraquat; RA, Retinoic acid; ROS, Reactive oxygen species; t-BHP, Tert-butyl hydroperoxide; TPA, 12-O-Tetradecanoylphorbol-13-acetate; ΔΨm, Mitochondrial membrane potential.

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The neurotoxicant 6-hydroxydopamine (6-OHDA) is a chemical discovered in the late 1950s and later proved to be specifically toxic to catecholaminergic neurons (Bastías-Candia et al., 2019), being a reference chemical to evoke PD in cultured cells and animal research. Structurally, it has some similarities with DA and noradrenaline (NA), which enables it to enter both dopaminergic and noradrenergic neurons. It has a high affinity for catecholaminergic membrane transporters, the DA-transporter (DAT) and NA-transporter (NAT), and inside the cells it is rapidly oxidized to hydrogen peroxide and paraquinone, which are highly toxic to mitochondria by specifically affecting complex I (Blandini & Armentero, 2012; Bové et al., 2005).

Occupational exposure to the potent herbicide paraquat (1,1′-dimethyl-4-4′-bipyridinium dichloride, PQ), in addition to its documented acute toxicity to other organs, brought a new focus on its potential as an inducer of PD (Baltazar et al., 2014; Gorell et al., 1998; Hughes, 1988). Given the growing evidence for health risks related to PQ, many countries, including the European Union, have banned paraquat use. However, in the United States, from 2013 to 2018, its sales more than doubled (Dorsey & Ray, 2023). Not only farmers and families are exposed to PQ, but also people from rural areas due to the contaminated air and water. The structural similarity between PQ and 1-methyl-4-phenylpyridinium (MPP^+) allows it to cross through the blood–brain barrier (BBB), affecting the dopaminergic system via DAT (Corasaniti et al., 1998) and the striatal cells (Shimizu et al., 2003). Although the mechanism behind PQ-induced dopaminergic neurotoxicity remains to be fully elucidated (Martins et al., 2013), mechanisms such as excitotoxicity, an increase in reactive oxygen species (ROS) production, and inhibition of mitochondrial complex I have been demonstrated (Bagchi et al., 1995; Shimizu et al., 2003; Tawara et al., 1996). When comparing the two toxicants, PQ may represent several advantages over 6-OHDA for studying PD mechanisms *in vitro*, due to its high stability when in solution, whereas 6-OHDA is more photosensitive and may lose activity when in solution for long exposure periods (Requejo et al., 2020).

Amphetamine (AMPH) and methylphenidate (MPH) are two stimulant drugs used in the treatment of attention-deficit hyperactivity disorder (Markowitz & Patrick, 2017; Volz, 2008). Meanwhile, they are also being studied as potential neuroprotectors regarding several CNS diseases. AMPH and MPH treatment have been linked to increased neuronal plasticity, enhanced neuronal growth, decreased oxidative stress, improved motor function and increased expression of growth-associated protein 43 (GAP-43) and synaptophysin (relevant proteins in the identification of axonal sprouting and synaptogenesis) in a CNS injured condition (Griesbach et al., 2008; Papadopoulos et al., 2009; Rasmussen et al., 2006; Stroemer et al., 1998). Moreover, several clinical trials suggested AMPH and MPH could be valuable treatments for stroke, traumatic brain injury, and some PD symptoms, due to the improvement in activities of daily living, memory, motor and cognitive functions (Crisostomo et al., 1988; Grade et al., 1998; Gualtieri & Evans, 1988; Kim et al., 2006; Lokk et al., 2011; Mooney & Haas, 1993; Pollak et al., 2007; Walker-Batson et al., 1995). AMPH and MPH increase the monoamine levels at the synaptic cleft, which may be key for the protective actions seen. Structurally similar to NA, DA, and serotonin (Carvalho et al., 2012; Heal et al., 2013), AMPH is an indirect monoamine agonist capable of inhibiting monoamine reuptake, vesicular monoamine transporter-2 (VMAT-2) and monoamine oxidase (MAO). It also promotes monoamine reverse transport, which results in monoamine release from presynaptic terminals in the CNS and periphery (De La Torre et al., 2004; Heal et al., 2013). MPH acts as a DAT and NAT inhibitor, namely in the prefrontal cortex and striatum, by blocking DAT and NAT in the presynaptic cell membrane (Volz, 2008). Likewise, it promotes VMAT-2 redistribution, as the subcellular distribution of VMAT-2-containing synaptic vesicles are differentially and selectively changed within nerve terminals (Sandoval et al., 2002; Zheng et al., 2006).

There are several cellular models to evaluate PD *in vitro*. The use of neuronal cell lines has several advantages over the use of primary human or animal tissues that in comparison have limited proliferative capacity, are heterogeneous, and involve ethical concerns. Cell lines offer the possibility of many manipulations, are proliferative, and can be genetically manipulated, at a lower cost with few regulatory issues. The use of patient-induced pluripotent stem cells (iPSCs) is a more recent option to study PD *in vitro*, as they can generate DA neurons from patients carrying mutations in the PD genes (Bose et al., 2022). The human neuroblastoma SH-SY5Y cell line is frequently used to model PD *in vitro* (Krishna et al., 2014) due to its ability to differentiate to the dopaminergic phenotype (Xicoy et al., 2017). Other neuroblastoma cell lines have been established with different properties than SH-SY5Y cells, however, this remains one of the most widely used cell lines to model PD *in vitro* (Carvajal-Oliveros et al., 2022).

Taking all the above into account, the present study aimed to evaluate *in vitro* the neuroprotective potential of AMPH and MPH, using clinically meaningful concentrations, against two well-characterized dopaminergic neurotoxicants, 6-OHDA and PQ, in differentiated human dopaminergic SH-SY5Y cells.

2. Material and methods

2.1. Materials

Sterile pipettes were obtained from Nerbe plus (Winsen, Germany), 48-well plates from TPP (Trasadingen, Switzerland), and 25 cm^3 flasks from Corning (New York, USA). Phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} was obtained from Biochrom (Berlin, Germany), antibiotic (10.000U/ml Penicillin / 10 mg/ml Streptomycin), heat-inactivated fetal bovine serum (FBS) and Hanks' balanced salt solution (HBSS) with Ca^{2+} and Mg^{2+} were obtained from Pan Biotech (Aidenbach, Germany). Dulbecco's modified Eagle's medium (DMEM) high glucose, neutral red (NR) solution (0.33 % *w/v*), trypan blue solution (0.4 % *w/v*), trypsin-EDTA solution, sodium bicarbonate, dimethyl sulfoxide (DMSO), PQ, 6-OHDA hydrochloride, *tert*-butyl hydroperoxide (t-BHP) solution, retinoic acid (RA), 12-O-tetradecanoylphorbol-13-acetate (TPA), carbonyl cyanide m-chlorophenyl hydrazone-1 (CCCP-1), bisBenzimide thrihydrochloride (Hoescht dye) and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were obtained from Merck (St. Louis, USA). Acetic acid glacial (97 %) was obtained from Fisher Chemical (Loughborough, UK), and alcohol (99.9 %) was obtained from Fábrica do álcool (Torres Novas, Portugal). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (98 %) was obtained from Alfa Aesar (Kandel, Germany). Threo-methyl-α-phenyl-2-piperidineacetate hydrochloride (MPH) and AMPH were obtained from Tocris (Bristol, United Kingdom). Tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was obtained from Abcam (Cambridge, UK).

2.2. Methods

2.2.1. Cell culture experimental protocols

SH-SY5Y cells [European Collection of Authenticated Cell Cultures (ECACC)] were obtained from Sigma-Aldrich (St. Louis, USA), and cultured in 25 cm^3 flasks. Cells were grown to confluence in DMEM medium supplemented with 10 % (*v/v*) FBS plus 1 % (*v/v*) of antibiotic. Cells were incubated in a humidified atmosphere of 5 % CO_2 – 95 % air at 37 °C. Cells were used between passages 18 and 30 for the experiments. When confluent, the cells were dissociated with trypsin–EDTA solution and sub-cultured in 48 well-plates. Cells were seeded (density of 25 000 cells/cm^2) and exposed initially to RA (10 nM) for 3 days, and later to TPA (80 nM) for another 3 days, to achieve neuronal differentiation and a dopaminergic phenotype (Ferreira et al., 2013). After six days, the medium was replaced with a new DMEM medium, and the cells were exposed to the drugs and/or toxicants of interest for 24 hours.

2.3. Cytotoxicity evaluation

After the differentiation protocol, cells were exposed to AMPH or MPH (concentration ranges 0.001–10.0 μM), 6-OHDA (concentration ranges 31.25–500 μM), or PQ (concentration ranges 100–5000 μM) for 24 hours. The concentrations of toxicants were selected to provide a complete concentration toxicity curve and disclose the pattern of toxicity associated with each toxicant. However, we acknowledge that the high concentrations used might not correlate to the human scenario of exposure, mainly in the case of paraquat. For instance, for a high survival probability, intoxicated paraquat patients should have a blood concentration of 1 mg/L (~4μM) at 1-hour post-exposure (Hart et al., 1984), therefore significantly below the concentrations tested in our study. From a mechanistic perspective, however, it is important to establish this curve to select adequate cytotoxic concentrations to engage in neuroprotection experiments.

All the drugs were solubilized in deionized H_2O and stored at −20 °C. However, due to their chemical instability, 6-OHDA solution was prepared fresh before each use. To avoid any solvent influence, the control wells received the same volume of sterile H_2O as the drug/toxicant treatment wells. NR uptake and MTT reduction assays were conducted after the exposure period to determine overall cytotoxicity.

2.3.1. MTT reduction assay

MTT assay is a colorimetric assay that reflects mainly the activity of mitochondrial enzymes (Mosmann, 1983). Following a 24-hour exposure to the compounds under study, 25 μL of MTT (5 mg/mL) was added to each well. After a 1.5-hour incubation period at 37 °C, 5 % CO_2 the medium was removed, and the formed formazan crystals were dissolved in 200 μL of DMSO. The plates were placed in a microplate shaker protected from light, for 15 min, to obtain a homogeneous solution. The formazan absorbance was read at 570 nm in a microplate reader (Biotech Synergy HT, Vermont, USA), whose value was subtracted from the values obtained in the reference wavelength of 690 nm. The results are expressed as the percentage of control, which was set to 100 %. Assays were done in quadruplicate per independent experiment.

2.3.2. Lysosomal NR uptake assay

The NR assay is one of the most widely used cytotoxicity tests and is based on the NR dye incorporation into lysosomes of viable cells (Repetto et al., 2008). After 24-hour exposure to the tested compounds, the medium was discarded, and 250 μL of warm culture medium supplemented with NR (33 μg/mL) was added to each well. After a 1.5-hour incubation at 37 °C, 5 % CO_2, the medium was removed, and 250 μL of warmed HBSS with Ca^{2+} and Mg^{2+} was added to each well. Then, the HBSS solution was removed, and 200 μL of solubilization solution (1 % acetic acid/50 % ethanol) was added to each well to extract the NR dye retained inside cells. The plate was carefully shaken in a microplate shaker while being kept out of the light until obtaining a homogeneous solution. The absorbance was measured at 540 nm and 690 nm (reference wavelength) in a microplate reader (Biotech Synergy HT, Vermont, USA). The results are expressed as the percentage of control, which was set to 100 %. In each independent experiment, the assays were performed in quadruplicate.

2.4. Cells' microscopic evaluation

2.4.1. Phase contrast microscopy

After a 24-hour exposure period to 6-OHDA (125 μM) or PQ (500 μM), morphological changes in SH-SY5Y differentiated cells were assessed by capturing phase-contrast microphotographs in a Nikon Eclipse TS100 equipped with a Nikon DS-Fi1 camera (Japan).

2.4.2. Hoechst nuclear staining

The effects of neurotoxicants on differentiated SH-SY5Y cells' nuclear morphology were evaluated by Hoechst staining after 24-hour exposure to 6-OHDA (125 μM) or PQ (500 μM). Briefly, cells were stained with the nuclear dye Hoechst 33,342 (10 μM) for 10 min at 37 °C, protected from light. Then, the cells were observed and microphotographed in a microscope Nikon Eclipse TS100 equipped with a Nikon DS-Fi1 camera (Japan) using a fluorescent filter (λexcitation maximum = 346 nm and λemission maximum = 460 nm).

2.5. Neuroprotection experiments

For neuroprotection experiments, cells were preincubated with AMPH (1 μM) or MPH (1 μM) for 30 min before exposure to toxicants. After this period, toxicants were added at concentrations that were selected following analysis of the concentration toxicity curves (62.5 and 125 μM for 6-OHDA, or 300 and 500 μM for PQ). The NR uptake and the MTT reduction assays were carried out as described above.

2.6. Mitochondrial membrane potential assay

The cyanine dye JC-1 has been used to detect the mitochondrial membrane potential (ΔΨm) created by the electrochemical gradient of the mitochondrial electron transport chain (Sakamuru et al., 2016; Sivandzade et al., 2019). Differentiated cells were exposed to AMPH (1 μM) or MPH (1 μM), 6-OHDA (62.5 or 125 μM) or PQ (300 or 500 μM), or CCCP-1 (100 μM), which is a positive control for mitochondrial depolarization. JC-1 was added to each well for the evaluation of ΔΨm, following 24-hour exposure to drugs. After JC-1 incubation (20 μM), the plate was gently mixed. Following the incubation period of 15 min, a centrifugation step was carried out for 5 min at 400*g*. The medium was removed and replaced with 400 μL of warm HBSS with Ca^{2+} and Mg^{2+}. This step was repeated once. Next, fluorescence was measured in a microplate reader (Biotech Synergy HT, Vermont, USA). The results were determined as the ratio between aggregate form (λexcitation = 535 nm and λemission = 595 nm) and monomer conformation (λexcitation = 485 nm and λemission = 535 nm) and expressed as a percentage of the control cells.

2.7. Reactive oxygen species levels evaluation by the DCFH-DA assay

The DCFH-DA probe was used to evaluate intracellular ROS production at several time points. Differentiated SH-SY5Y cells were incubated with DCFH-DA 200 μM at 37 °C, 5 % CO_2 for 30 min, protected from light. Following the pre-incubation phase, the media was replaced with 250 μL of a new warm cell culture medium. The cells were then exposed to AMPH (1 μM) or MPH (1 μM) at 37 °C. Thirty minutes later, the neurotoxicants PQ or 6-OHDA were added. As a positive control for ROS production, 200 μM *tert*-butyl hydroperoxide was used. Afterward, the fluorescence was measured in a microplate reader (Biotech Synergy HT, Vermont, USA) with the excitation and emission wavelengths of 485 nm and 530 nm, respectively, at the time points 0, 1, 2, 3, 4, 5, 6, and 24 hours. In each independent experiment, the assays were performed in triplicate.

2.8. Statistical analysis

Results are presented as mean ± standard error (SD), except for concentration-toxicity curves, which were presented as mean and 95 % confidence interval (CI 95 %). The data distribution normality was evaluated by four tests: Anderson-Darling test, D'Agostino & Pearson test, Shapiro-Wilk test, and Kolmogorov-Smirnov test. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test, once data followed a parametric distribution confirmed by at least one normality distribution test. Regarding ROS production results, two-way ANOVA repeated measurements were performed, followed by Tukey's multiple comparisons test. For 6-OHDA and PQ, the lethal concentration 50 % (LC50) or 25 % (LC25) figures were determined by analysis of the MTT and NR concentration-toxicity

curves. Curves were fitted using least squares as the fitting method. Statistical significance was considered with a $p < 0.05$. All statistical analyses were performed using the GraphPad Prism 8.3.0 software (San Diego, CA, USA).

3. Results

3.1. Neither AMPH nor MPH caused any cytotoxicity or changed the mitochondrial membrane potential

AMPH and MPH therapeutic-relevant concentrations were screened for potential cytotoxicity to select a working concentration for use in later studies. At 24 hours, in the MTT reduction and NR uptake assays, neither AMPH nor MPH caused significant cytotoxicity when compared to controls (Supplementary Fig. 1A-D), under the concentration range used (0.001–10 μM). Further evaluation with the JC-1 probe revealed no significant alterations in the ΔΨm following a 24-hour exposure of differentiated SH-SY5Y cells to 1 μM of AMPH or MPH when compared to controls (Supplementary Fig. 1E). Therefore, to conduct neuroprotection experiments the working concentration for both AMPH and MPH was set to 1 μM.

3.2. 6-OHDA led to concentration-dependent neurotoxicity and decreased mitochondrial membrane potential

Significant cytotoxicity was seen in the NR uptake assay at 24 hours, starting with the concentration of 62.5 μM of 6-OHDA, compared to the control (Fig. 1A). This result highlights concentration-dependent cytotoxicity induced by 6-OHDA in the NR uptake assay. At the same time point, 6-OHDA induced a similar response with a decrease in the cell's MTT reduction ability (Fig. 1B) from the 62.5 μM concentration onwards. In the NR uptake assay, 6-OHDA exhibited concentration-dependent cytotoxicity, which was consistent with earlier findings. Turning now to the mitochondrial membrane potential assessment (Fig. 1C), after a 24-hour exposure, the JC-1 probe revealed a steep decrease of ΔΨm induced by both 6-OHDA concentrations (62.5 and 125 μM) under study when compared to controls. This result proves a concentration-independent ΔΨm decrease.

3.3. PQ neurotoxicity was concentration-dependent and accompanied by a decrease in mitochondrial membrane potential

The one-way ANOVA revealed a significant decrease in the NR uptake assay after 24-hour exposure to 200 μM or more of PQ when compared to the control cells (Fig. 2A). Regarding the MTT reduction assay (Fig. 2B), a 24-hour PQ exposure to SH-SY5Y cells induced a significant concentration-dependent decrease, with the highest tested concentration of PQ being the most cytotoxic. If we now turn to the mitochondrial membrane potential evaluation, the highest PQ concentration tested (500 μM) induced a significant decrease of the ΔΨm after 24-hour exposure, when compared to control cells (Fig. 2C).

3.4. Estimating LC25 and LC50 concentrations for 6-OHDA and PQ

As observed in the MTT reduction and NR uptake assays, concentration-dependent toxicity was induced by 6-OHDA and PQ in differentiated SH-SY5Y cells. Therefore, the LC25 (25 % of lethality) and LC50 (50 % of lethality) figures for both cytotoxicity assays at 24-hour exposure were calculated through fitted concentration-toxicity curve analysis. Calculated values are presented in Table 1. For the MTT reduction assay, while 6-OHDA had an LC25 of 99.9 μM and an LC50 of 133.6 μM, PQ showed an LC25 of 422 μM and an LC50 of 585.8 μM. Regarding data from the NR uptake assay, 6-OHDA LC25 was 73.1 μM while the LC50 was 105.7 μM, and PQ LC25 was 368.2 μM while the LC50 was 526.7 μM. While for 6-OHDA, the toxicity was very steep and LC25 and LC50 were very close in both assays. However, with an exponential increase in toxicity, we had to make compromises. We selected a concentration near the LC50 for both assays (125 μM) and

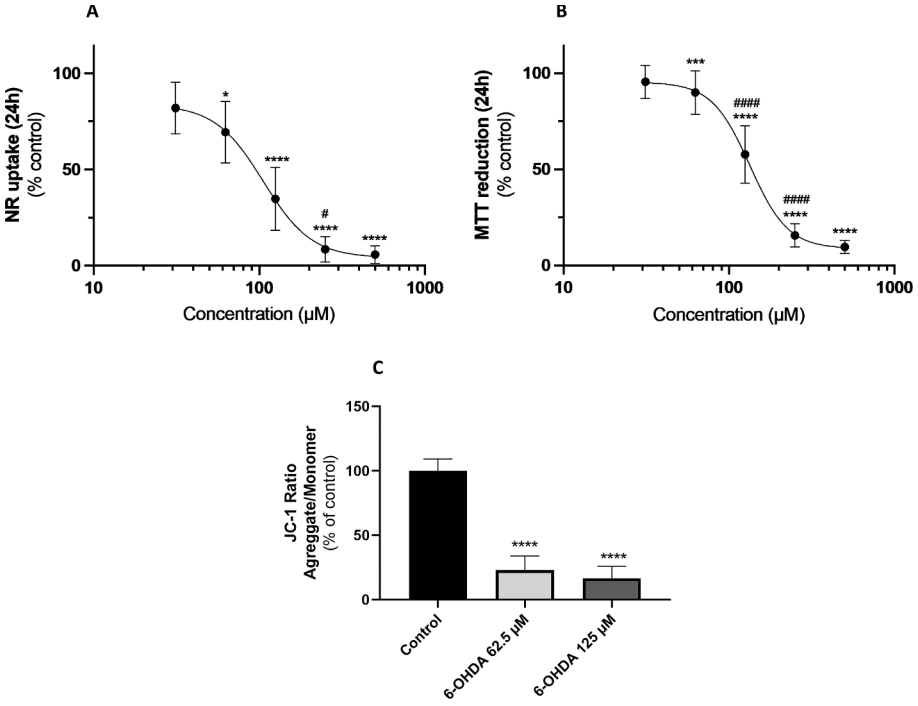

Fig. 1. Concentration-response curves for 6-OHDA. Differentiated SH-SY5Y cells were exposed to 31.25, 62.5, 125, 250, and 500 μM of 6-OHDA for 24 hours and the NR uptake (A) and the MTT reduction (B) assays were performed. Results are presented as mean ± SD (N = 24 to 28 different wells from 7 independent experiments). Mitochondrial membrane potential evaluated by JC-1 probe in differentiated SH-SY5Y cells incubated with 62.5 or 125 μM of 6-OHDA (C) after 24-hour exposure. Results are presented as mean ± SD (N = 6 different wells from 2 independent experiments). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test (*$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$ vs. Control; $^{\#}p < 0.05$, $^{\#\#\#\#}p < 0.0001$ vs. previous lower concentration tested).

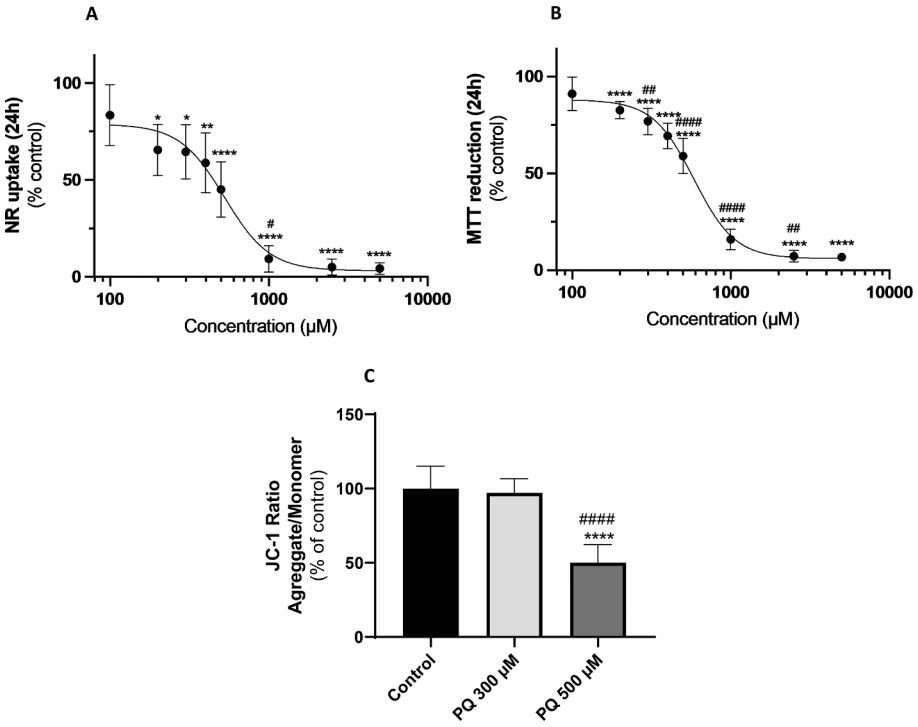

Fig. 2. Concentration-response curves for PQ. Differentiated SH-SY5Y cells were exposed to 100, 200, 300, 400, 500, 1000, 2500, and 5000 μM of PQ for 24 hours and the NR uptake (A) and MTT reduction (B) assays were performed. Results are presented as mean ± SD (N = 16 to 43 different wells from 4 to 7 independent experiments). Mitochondrial membrane potential evaluated by JC-1 probe in differentiated SH-SY5Y cells incubated with 300 or 500 μM of PQ (C) after 24-hour exposure. Results are presented as mean ± SD (N = 6 different wells from 2 independent experiments). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test (*$p < 0.005$, **$p < 0.01$, **** $p < 0.0001$ vs. Control; $^{\#}p < 0.05$, $^{\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ vs. previous lower concentration tested).

Table 1
LC25 and LC50 of PQ and 6-OHDA.

	PQ		6-OHDA	
	NR uptake assay	MTT reduction assay	NR uptake assay	MTT reduction assay
LC_{25} [μM]	368.2	422	73.1	99.9
LC_{50} [μM]	526.7	585.8	105.7	133.6

Estimates of LC25 (Lethal concentration 25 %) and LC50 (Lethal concentration 50 %) after 24-hour exposure, obtained by analysis of the MTT and NR concentration-toxicity curves. Curves were fitted using least squares as the fitting method. The LC25 and LC50 data are presented in μM.

used half that concentration to study a low toxicity response (62.5 μM), but a truly cytotoxic concentration as seen in the data of the MTT reduction assay. On the other hand, the required LC50 PQ concentration in the NR uptake assay was almost double that of the LC25, hence working concentrations chosen were near those, 300 μM or 500 μM of PQ.

3.5. Both 6-OHDA and PQ led to cellular damage with signs of apoptosis

After a 24–hour exposure to 6-OHDA (125 μM) or PQ (500 μM), the morphological assessment by phase-contrast microscopy and Hoescht staining was done, to ascertain the microscopic features associated with neurotoxicants-induced cytotoxicity. Cellular death was accompanied by dendrite loss in differentiated SH-SY5Y cells (Fig. 3). What stands out in the micrographs of SH-SY5Y cells exposed to 6-OHDA or PQ stained with Hoechst 33,342 (Fig. 3D and 3F) is the chromatin condensation (green arrows) and the presence of pyknotic nuclei (red arrows). Those features were virtually absent in control cells.

3.6. Neither AMPH nor MPH prevented any features of 6-OHDA toxicity

A significant decrease was seen in cells exposed to 6-OHDA (125 μM) in the NR uptake assay as expected, but this cytotoxicity was not prevented by pre-incubation with AMPH 1 μM or MPH 1 μM (Fig. 4A). The lack of protection similarly was not observed for the 62.5 μM concentration (data not shown). Accordingly, pre-incubation with AMPH 1 μM or MPH 1 μM (Fig. 4B) did not prevent the cytotoxicity afforded by 6-OHDA in the MTT reduction assay. Pre-incubation with AMPH 1 μM or MPH 1 μM did not significantly protect differentiated SH-SY5Y cells from the decrease in ΔΨm caused by 6-OHDA (125 μM) exposure, according to JC-1 probe assay (data not shown).

3.7. ROS production induced by 6-OHDA was not prevented by AMPH or MPH

The fluorescent probe DCFH-DA was used to measure ROS production (Fig. 5). At 125 μM of 6-OHDA, SH-SY5Y cells produced significantly more ROS than the control group only after 24 hours. On the other hand, a time-persistent significant increase was induced by 1 μM of AMPH when compared to the control cells at all time points. Also, the combination of 1 μM of AMPH and 125 μM of 6-OHDA showed a significant increase in ROS production at the 24-hour time-point, similar to levels of 6-OHDA alone. MPH induced a significant ROS production in differentiated SH-SY5Y cells at 0, 1, 2, 4, 6, and 24 hours when compared to control cells. Meanwhile, when the pre-incubation with MPH was followed by 6-OHDA (125 μM), a significant increase in ROS production was also seen at 24-hour, similar to levels of 6-OHDA alone. Overall, these results indicate that 6-OHDA (125 μM) induced an increase in ROS levels, that was not prevented by the tested neuroprotectors. Also, AMPH and MPH alone can induce low, but statistically significant, levels of ROS. To validate our experimental method, t-BHP

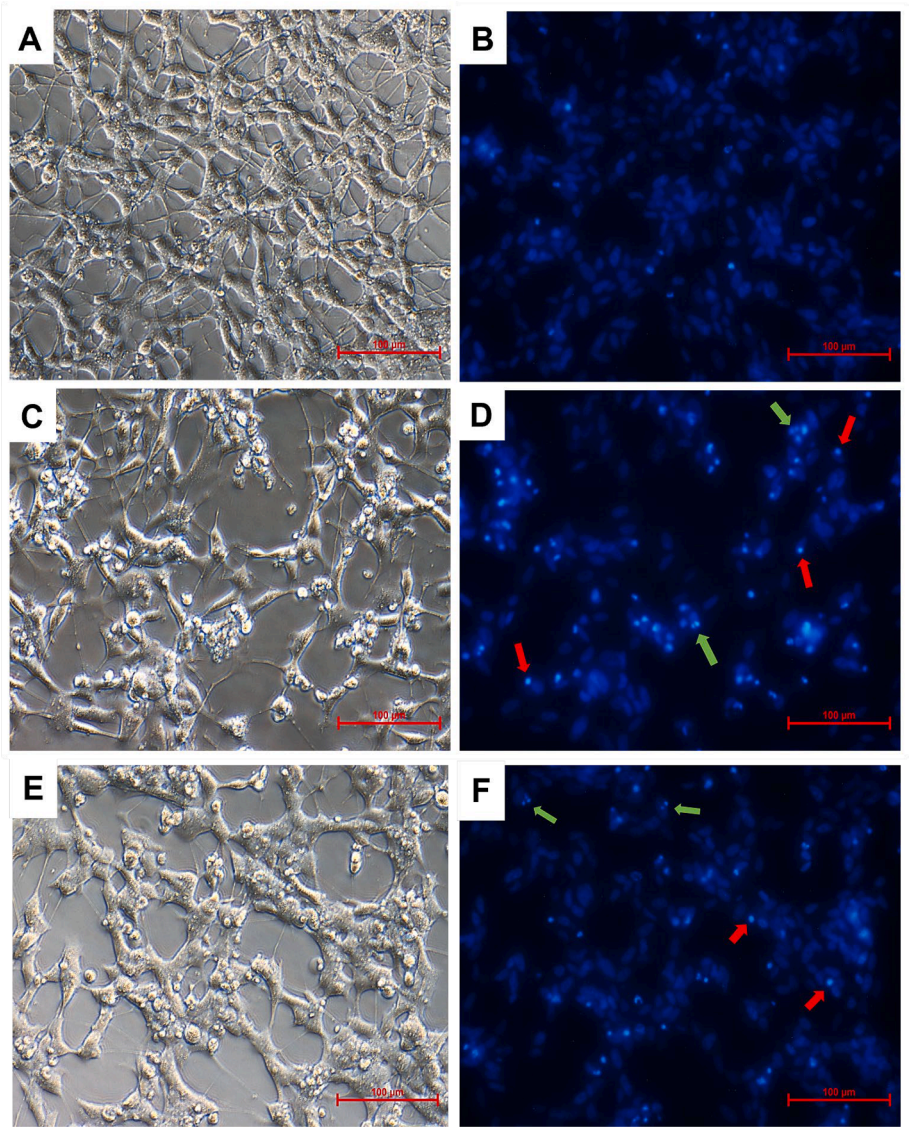

Fig. 3. Morphological analysis by phase contrast microscopy (left side) of differentiated SH-SY5Y cells exposed for 24 hours to 6-OHDA 125 µM (C) or PQ 500 µM (E), and the control group (A). Microphotographs from Hoechst nuclear staining (right side) were taken after a 24-hour exposure period to 6-OHDA 125 µM (D) or PQ 500 µM (F), and control group (B). Inserted red arrows point to the pyknotic nuclei, while green arrows indicate chromatin condensation. Representative micrographs were taken of randomly chosen fields in 48-well plates (scale bar 100 µm) from 3 independent experiments.

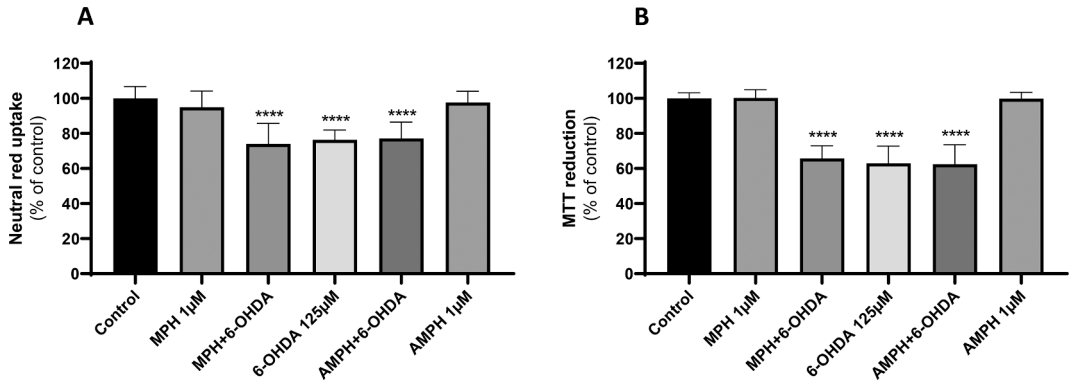

Fig. 4. Cellular cytotoxicity evaluated by the NR uptake (A) and MTT reduction (B) assays in differentiated SH-SY5Y cells pre-incubated with 1 µM of AMPH or MPH and then exposed to 125 µM of 6-OHDA for 24 hours. Results are presented as mean ± SD (N = 13 to 16 different wells from 4 independent experiments). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test (****$p < 0.0001$ vs. Control).

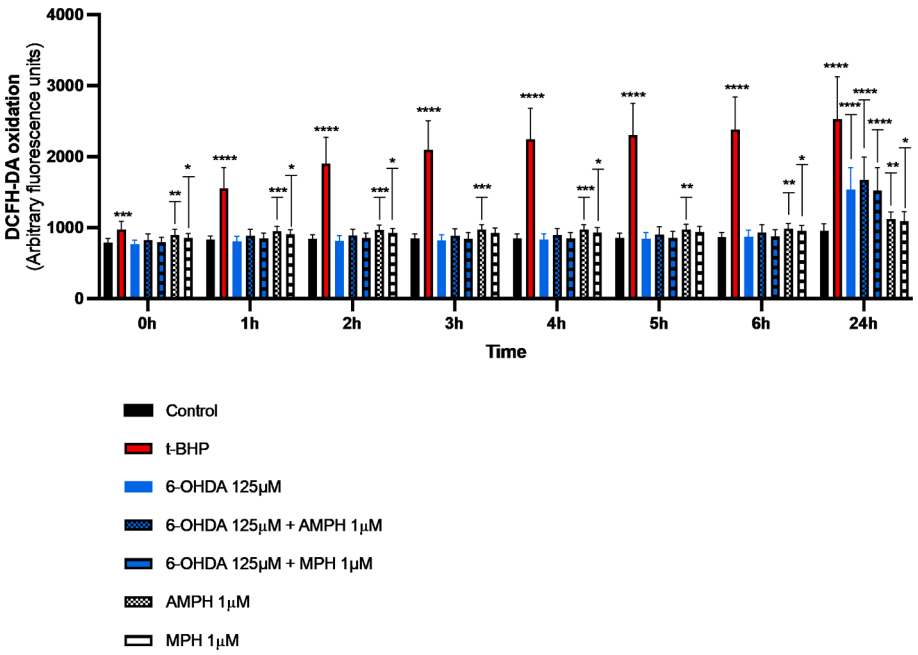

Fig. 5. Assessment of reactive oxygen species (ROS) production, using the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) probe, on differentiated SH-SY5Y cells exposed to 6-OHDA 125 μM, AMPH 1 μM, MPH 1 μM and 6-OHDA 125 μM pre-incubated with AMPH 1 μM or MPH 1 μM until 24-hour exposure. Results are presented as mean ± SD (N = 15 different wells from 5 independent experiments). Statistical analysis was performed using two-way ANOVA repeated measurements followed by Tukey's multiple comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001 ****p < 0.0001 vs. Control).

(200 μM) was used as a positive control, resulting in impressive ROS generation across all time points tested. Of note, we also evaluated the ability of 62.5 μM 6-OHDA to induce ROS and verified that this concentration also led to increased ROS at the 24-hour time-point (data not shown).

3.8. AMPH and MPH partially protected differentiated SH-SY5Y cells against PQ-induced neurotoxicity

Concerning PQ in the NR uptake assay, at 24-hour exposure, both PQ concentrations (300 μM and 500 μM) induced significant cytotoxicity compared with the control conditions. Nevertheless, that cytotoxicity was not prevented by the pre-incubation with AMPH (1 μM) or MPH (1 μM) (Fig. 6A and 6B). In the case of the MTT reduction assay, both PQ concentrations (300 μM and 500 μM) induced a significant decrease in MTT reduction ability when compared with the control conditions at 24-hour. However, the cytotoxicity induced by PQ (300 μM) was partially prevented by the pre-incubation with either AMPH (1 μM) or MPH (1 μM) (Fig. 6C). Meanwhile, the cytotoxicity induced by the highest PQ concentration (500 μM) only was partially prevented by the pre-incubation with MPH 1 μM, but not by the pre-incubation with AMPH 1 μM (Fig. 6D). Overall, these results show that in the MTT reduction assay, both AMPH and MPH partially protected cells from the cytotoxicity induced by PQ 300 μM. At the same time, MPH was responsible for the partial protection against the cytotoxicity induced by PQ 500 μM. If we now turn to the mitochondrial membrane potential, in cells pre-incubated with AMPH 1 μM or MPH 1 μM and then exposed to PQ (500 μM) it was observed that the decrease of ΔΨm relative to controls was lower, when compared to cells only exposed to 500 μM of PQ, ruling for a partial neuroprotective effect of AMPH and MPH (Fig. 6E). To validate our experimental method, CCCP (100 μM) was used as a positive control, causing mitochondrial membrane potential depolarization (Fig. 6E).

It is worth mentioning that although we tested the ability of PQ to evoke ROS production, we did not see any increase in ROS levels at any of the time points tested in our differentiated cellular model (data not shown). As such, we did not test the neuroprotector's action against PQ toxicity in this experimental paradigm.

4. Discussion

This study revealed the following major findings: (1) therapeutic concentrations of AMPH and MPH did not induce cytotoxicity or mitochondrial membrane potential changes to differentiated SH-SY5Y cells after a 24-hour exposure; (2) 6-OHDA induced concentration-dependent neurotoxicity, increased ROS production, and decreased mitochondrial membrane potential; (3) PQ induced concentration-dependent neurotoxicity and its highest concentration led to a significant decrease in mitochondrial membrane potential in differentiated SH-SY5Y cells, although it did not induce ROS production; (4) Both 6-OHDA and PQ led to cellular damage with signs of apoptosis in differentiated SH-SY5Y cells; (5) AMPH and MPH did not prevent 6-OHDA toxicity in differentiated SH-SY5Y cells; (6) AMPH and MPH partially protected differentiated SH-SY5Y cells against PQ-induced neurotoxicity.

Differentiated SH-SY5Y cells are suitable for studying AMPH and MPH psychostimulant effects since their target is the dopaminergic transporter (Carvalho et al., 2012). The clinical use of MPH and AMPH typically involves oral administration of relatively low doses, and concentrations of 1 to 100 nM were found in patients' serum upon 1 mg/kg MPH oral intake (Grünblatt et al., 2018). Meanwhile, blood concentrations of 10 nM–1 μM correlate well with mixed AMPH salt extended-release formulation of 30 mg administration (Biederman et al., 2007). Therefore, we selected a range of clinically relevant concentrations of 0.001, 0.01, 0.1, 1, and 10 μM of both AMPH and MPH in an attempt to mimic the low concentrations attained in human plasma. It is worth noting that these concentrations are significantly lower than the AMPH 2 mM concentration that caused cytotoxicity in differentiated SH-SY5Y cells, as evaluated by the MTT assay (Feio-Azevedo et al., 2017). Moreover, a previous study showed neurite outgrowth promotion by MPH at these concentration ranges in differentiated SH-SY5Y cells (Grünblatt et al., 2018).

We found neither cytotoxicity nor ΔΨm changes in neuronal cells after exposure to both AMPH and MPH at these concentration ranges. These findings suggest that the tested concentrations do not appear to

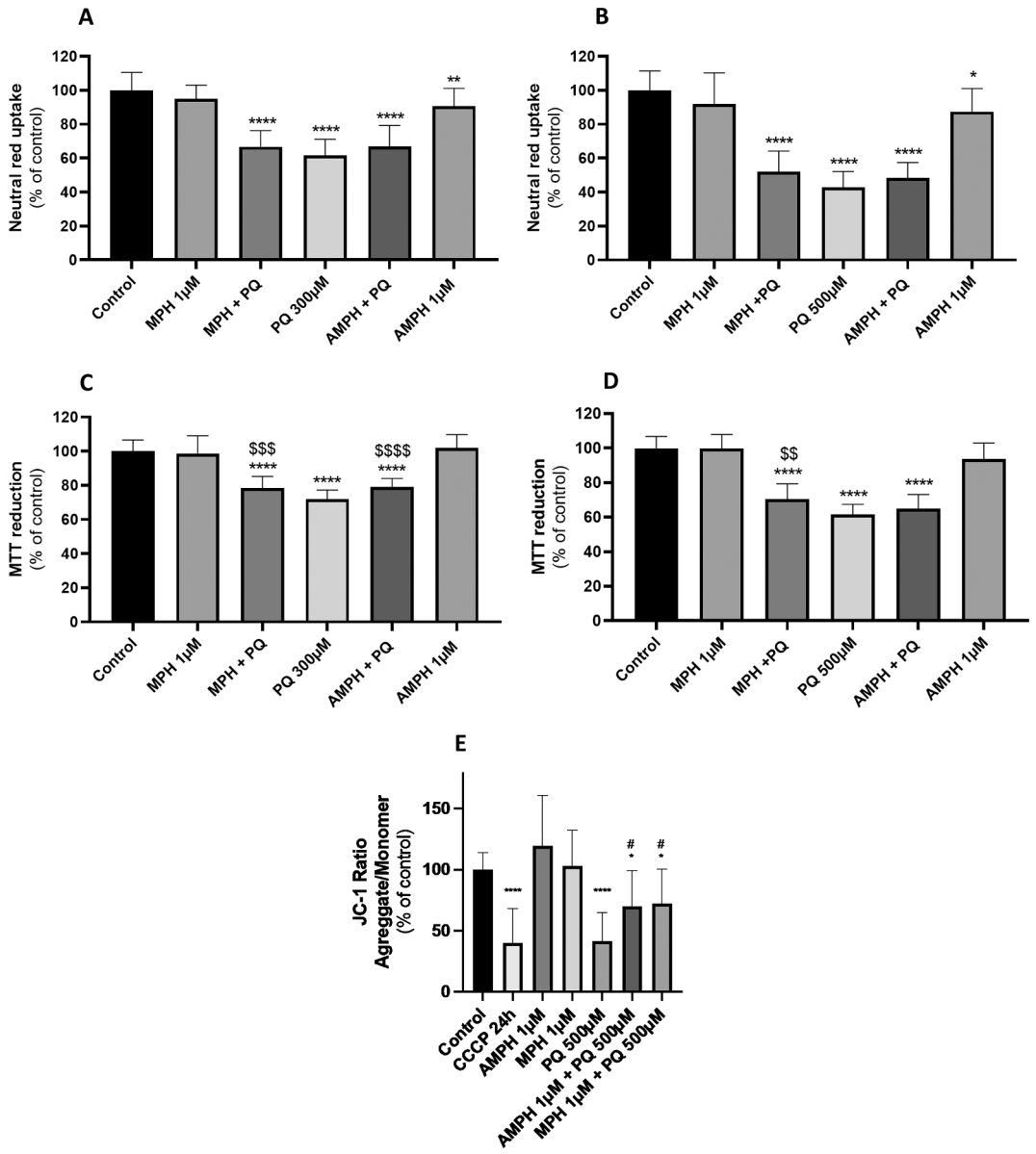

Fig. 6. Cellular cytotoxicity evaluated by the NR uptake and MTT reduction assays in differentiated SH-SY5Y cells pre-incubated with 1 μM of AMPH or MPH and then exposed to 300 μM (A, C) or 500 μM (B, D) of PQ for 24 hours. Results are presented as mean ± SD (N = 24 to 32 different wells from 6 (B, D) to 7 (A, B) independent experiments). Mitochondrial membrane potential evaluated by JC-1 probe in differentiated SH-SY5Y cells exposed to 500 μM of PQ (E) during 24 hours. Results are presented as mean ± SD (N = 18 to 24 different wells from 6 independent experiments). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison tests (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ vs. Control; $^{\$\$}p < 0.01$ vs PQ 500 μM; $^{\$\$\$}p < 0.001$, $^{\$\$\$\$}p < 0.0001$ vs PQ 300 μM; $^{\#}p < 0.05$ vs. PQ 500 μM).

cause mitochondrial dysfunction or damage in lysosomes. A normal ΔΨm is key to maintaining the function of the respiratory chain, along with maintaining the full potential in generating ATP, being usually a sign of healthy cells (Zorova et al., 2018). Thus, these clinically relevant concentrations of both AMPH and MPH are deemed non-toxic in several assays and can be tested *in vitro* as potential neuroprotectors.

Based on previous knowledge 6-OHDA and PQ are considered experimental toxicants to study the mechanisms of PD *in vitro* (Wen et al., 2020). The cytotoxic profile of 6-OHDA revealed a concentration-dependent pattern. These findings are consistent with those of Jordán and colleagues, who reported marked cytotoxicity induced by 6-OHDA in undifferentiated SH-SY5Y cells after exposure to concentrations ranging from 25 to 1000 μM for 24 hours, as assessed by the MTT reduction assay (Jordán et al., 2004). In addition, treatment of undifferentiated SH-SY5Y cells with 100 μM 6-OHDA resulted in morphological changes typical of apoptosis, revealing the same features that we report herein (Jordán et al., 2004). Another study in undifferentiated SH-SY5Y cells exposed to different concentrations of 6-OHDA (10–1000 μM) for 24 hours demonstrated concentration-dependent cytotoxicity when determined by the MTT reduction assay, and the LC50 was estimated to be 116.7 μM, which is in line with our results (Silva et al., 2018).

The mitochondria depolarization induced by 6-OHDA (62.5 and 125 μM), which we report corroborates the results of Silva and co-workers, who observed in SH-SY5Y undifferentiated cells that 6–OHDA (100 μM for a 3-hour exposure) evoked mitochondria depolarization (Silva et al., 2018). Our microscopic observations are consistent with the possibility that the collapse of the ΔΨm can initiate events like apoptosis. We also saw a significant increase in ROS levels after a 24-hour exposure to 6-OHDA. The loss of ΔΨm and ROS accumulation

are probably linked and can explain the apoptotic fate of cells exposed to 6-OHDA.

On the other hand, PQ's cytotoxic profile demonstrated concentration-dependent neurotoxicity. Using the same *in vitro* model, Martins *et al.* did not observe cytotoxicity after 24-hour exposure to PQ on the lactate dehydrogenase leakage assay (Martins et al., 2013). However, in the MTT reduction assay mitochondrial dysfunction was observed after a 24-hour exposure to the concentrations of 500 μM or 1000 μM, and after a 48-hour exposure to the 100 μM concentration. These results led the authors to hypothesize that PQ toxicity was due to the disruption of the mitochondrial electron transport chain (Martins et al., 2013). Yang and colleagues found that 0.5 and 1 mM of PQ dramatically reduced cell viability to 56.2 % and 42.8 % of the control, respectively, after a 48-hour exposure, as measured by trypan blue exclusion (Yang & Tiffany-Castiglioni, 2005). Also, Fujimori *et al.* reported decreased cell viability of about 50 % measured after 24-hour exposure to PQ 500 μM in differentiated SH-SY5Y cells (Fujimori et al., 2012). These reports are consistent with our results (LC50 = 585 μM and 526 μM in MTT and NR assay, respectively). It should be emphasized that different cell lines appear to have varying susceptibilities to PQ-induced cytotoxicity. For instance, in Caco-2 cells (a human colorectal adenocarcinoma cell line) the LC50 was approximately 1000 μM after a 24-hour exposure in the MTT assay (Silva et al., 2011). Meanwhile, in RBE4 cells (immortalized rat brain microvessel endothelial cells) only concentrations higher than 2.5 mM revealed toxicity (Vilas-Boas et al., 2014). Therefore, it seems that SH-SY5Y cells present a higher susceptibility to PQ-induced toxicity. We report that lower PQ concentrations resulted in more cytotoxicity in the NR assay than in the MTT assay, indicating that the former is more sensitive.

Another important finding was the loss of ΔΨm induced by 500 μM of PQ, but not by 300 μM of PQ, suggesting a concentration-dependent mitochondrial membrane depolarization. PQ evoked loss of ΔΨm is a common feature of mitochondrial programmed cell death. A similar pattern was seen for 6-OHDA. Even so, we cannot overrule other forms of cell death in the process. Accordantly, previous studies also demonstrated that differentiated SH-SY5Y cells treated with 0.5 mM of PQ significantly reduced ΔΨm to 69.8 % and 54.3 % of controls after 24- and 48-hour exposure, respectively (Yang & Tiffany-Castiglioni, 2005). A possible explanation might be the inhibition of the mitochondrial complex I promoted by PQ (Yang & Tiffany-Castiglioni, 2005). Contrary to expectations, we did not find ROS production induced by PQ (300 and 500 μM) following 24-hour exposure in the DCFH–DA assay. Differently, another study found significantly increased ROS levels to 129.8 % and 151.1 %, respectively, compared to controls in differentiated SH-SY5Y cells after a 24- and 48-hour exposure to PQ 0.5 mM, in an assay where fluorescence was read in cell lysates and not directly in cells (Yang & Tiffany-Castiglioni, 2005). In addition, Xiuli *et al.* reported a dose-dependent ROS production increase after PQ (10 and 100 μM) exposure in immortalized human embryonic neural progenitor cells (hNPCs) (Chang et al., 2013). The lack of ROS evoked by PQ in our work might be due to the sensitivity of the assay or the use of low concentrations. PQ's ability to disrupt the mitochondrial electron transport chain and promote loss of ΔΨm might interfere with ROS production by the mitochondria (Zhang & Wong, 2021), however, we don't know the extent of that interference.

The 6-OHDA and PQ neurotoxicants may enter the cell via DAT and promote the selective degeneration of dopaminergic neurons (Wen et al., 2020), supporting the argument for using DAT-acting drugs. However, we saw that AMPH and MPH could only partially prevent PQ-induced neurotoxicity, failing to prevent the 6-OHDA-induced one. These conclusions are based on the results obtained only by the MTT reduction assay, suggesting differences among the sensitivity of the assays used herein. Therefore, it appears that AMPH and MPH protected against the mitochondrial dysfunction caused by PQ, but not by the lysosomal dysfunction evoked by PQ. In agreement with these results is the protection afforded by the psychostimulant agents against the loss of ΔΨm induced by PQ, meaning that the neurotoxicant actions are possibly related to the action on mitochondrial complex I reported earlier. We hypothesize that DAT inhibition is achieved by the neuroprotectors causing a decrease in toxicants' entry into the cell, partially preventing/blocking its toxic effects. This hypothesis may be supported by Martins and co-workers, who showed partial protection against PQ-induced neurotoxicity by the specific DAT inhibitor GBR 12909 in differentiated SH–SY5Y cells (Martins et al., 2013). In the same way, the literature reports another study using GBR12909, which verified complete protection of differentiated SH-SY5Y cells against the cytotoxicity of PQ (0.05–0.5 mM), and also partially attenuated toxicity for the 1 mM concentration (Yang & Tiffany-Castiglioni, 2005). It is important to note that the entry of PQ into the cell via DAT is still controversial. While some authors defend a PQ entrance into dopaminergic neurons through the DAT (Yang & Tiffany-Castiglioni, 2005), others as Richardson and co-workers stated that PQ-induced toxicity is independent of DAT expression (Richardson et al., 2005). Therefore, other explanations are plausible for the protection evoked by AMPH and MPH against PQ neurotoxicity, and the direct action of those drugs on mitochondria cannot be overruled.

A possible reason for the lack of protection given by AMPH or MPH against 6-OHDA-induced neurotoxicity could be the considerable ROS generation caused by 6-OHDA after a 24-hour exposure, leading to the inability of the putative neuroprotectors to prevent it (in fact, both MPH and AMPH can cause ROS production *per se*). Hence, it could be hypothesized that if the ROS production generated by 6-OHDA occurs outside of the cells, the protective action of AMPH or MPH on inhibiting DAT may not be relevant for preventing these oxidative stress events. PQ, on the other hand, causes mitochondrial membrane potential depolarization without producing significant ROS, allowing AMPH and MPH protective properties, either by direct DAT blocking or direct mitochondrial action.

This study has potential limitations. It was conducted in a single cellular model of dopaminergic neurons, though the neurotoxicant's actions in vivo are not limited to neuronal damage. Additionally, it was not performed a deep evaluation of the mechanisms involved in cell death. Other studies *in vitro* and in vivo have been conducted to examine the neurotoxic pathways elicited by PQ and 6-OHDA. In the Neruo-2a cell line, 3 h of exposure to PQ (1 mM) or 6-OHDA (25 μM) evoked an increase in apoptotic cells, accompanied by a loss in mitochondrial membrane potential and increases in ROS and NO formation (Menchinskaya et al., 2021). In rat adrenal pheochromocytoma (PC12) cells, 6-OHDA (100 mM) exposure for 24 hours evoked cellular apoptosis accompanied by caspase 3 increase and cytochrome *C* release (Olatunji et al., 2016). Also, the neurotoxicant promoted an increase in the malondialdehyde (MDA) content and ROS levels, and a decrease in superoxide dismutase and glutathione peroxidase activities (Olatunji et al., 2016), ruling for the involvement of oxidative stress as we reported in our model. In another study, SH-SY5Y cells exposure to PQ for 24 hours, evoked iron accumulation, lipid ROS production, and mitochondrial damage, which culminated in cell death by ferroptosis (Zuo et al., 2021). PD is known to cause abnormal mitochondrial dynamics, suggesting the likely involvement of disturbed mitochondrial fission/fusion (Zhao et al., 2017). These events were observed after PQ and 6-OHDA, where these neurotoxicants enhanced mitochondrial fission and caused mitochondrial dysfunction both in cultured cells (dopaminergic cell lines SN4741 or SH-SY5Y and primary rat cortical neurons) and mice (Xi et al., 2018; Zhao et al., 2017). Accordingly, Mfn2 overexpression or enhancement could protect neurons against neurotoxicant-induced neuronal death (Xi et al., 2018; Zhao et al., 2017). However, the damage caused by these toxicants is not limited to neurons, and glial cells were involved in the neurotoxic response to 6-OHDA and PQ. Male C57BL/6 J mice (8 weeks old) injected with 6-OHDA into the medial forebrain bundle showed consistent activation of astrocytes and microglia in the substantia nigra pars compacta and striatum at 7 weeks, indicating a long-term glial response in the nigrostriatal system (Cui

et al., 2024). Male C57BL/6 mice treated intraperitoneally with PQ 10 mg/kg twice a week for 6 weeks showed microglial activation and elevated TNF-α and IL-6 levels in the substantia nigra (Yang et al., 2022). These findings indicate that neuroinflammation is connected to the neurotoxic effects of 6-OHDA and PQ and goes beyond neuronal lesions.

In conclusion, the toxicants used to modulate PD *in vitro* induced concentration-dependent cytotoxicity accompanied by depolarization of the ΔΨm. At the same time, AMPH and MPH could afford protection against the dopaminergic toxicity evoked by PQ, which could have clinical relevance to PD. To fully understand the precise processes underlying this neuroprotective effect, more research is required.

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Author contributions

Patrícia Carneiro, Mariana Ferreira, Vera Marisa Costa and João Paulo Capela performed the experiments and organized data and graphs. Vera Marisa Costa and Félix Carvalho contributed to data interpretation and manuscript organization. João Paulo Capela conceptualized the experiments. All authors contributed to the text and reviewed the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crtox.2024.100165.

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