1-Methyl-4-Phenylpyridinium-Induced Death of Differentiated SH-SY5Y Neurons Is Potentiated by Cholesterol

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Keywords

Dopaminergic neurons · Cholesterol-induced neuronal death · Tyrosine hydroxylase · Cell viability · Mitochondrial membrane potential

Abstract

Background/Aims: Hypercholesterolemia is recently considered a risk factor for Parkinson's disease (PD), the most consistent neurodegenerative movement disorder. The study aimed to investigate the effect of exogenous cholesterol on 1-methyl-4-phenylpyridinium (MPP⁺) parkinsonian neurotoxin-induced cell death, loss of mitochondrial membrane potential, and dopaminergic deficiency in a cellular model of PD. **Methods:** Cholesterol (50 μ M) when added in the culture media alone or in combination with MPP⁺ was studied in SH-SY5Y neuroblastoma cells. There were 4 groups that were studied; SH-SY5Y cells treated with vehicle (control), cells that were treated with 1 mM MPP⁺ (MPP⁺), or cholesterol (chol) or both (M + chol). The loss of cell survival was

measured by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Dopamine depletion, microtubule-associated protein 2 (MAP-2), and tyrosine hydroxylase (TH)-positive neuronal loss were determined by HPLC-electrochemical detection and TH immunocytochemistry respectively. Mitochondrial membrane potential in cells stained by tetramethylrhodamine methyl ester dye was analysed by flow cytometry. **Results:** Cholesterol treatment potentiated a reduction of neuronal viability with loss of TH-positive neurons in cultures. MPP⁺-induced depletion of dopamine level in the post-mitotic MAP-2 immunoreactive neurons and loss of mitochondrial membrane potential were also heightened by cholesterol. **Conclusion:** Apparently, changes in neuronal cholesterol content significantly influenced the neurotoxicity and the direct mitochondrial mechanisms involved in MPP⁺-induced cell death. Our observations demonstrate that high cholesterol incorporated into the differentiated human neuroblastoma cells worsened dopaminergic neuronal survivability through increased depolarization of mitochondrial membrane poten-

tial, which is a known mechanism of dopaminergic cell death by MPP⁺. The present findings support the hypothesis that hypercholesterolemia could be a risk factor for PD.

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Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by the irreversible loss of dopaminergic neurons in the substantia nigra pars compacta of the midbrain, depletion of dopamine (DA) in the striatum, and presence of intracellular Lewy bodies, which are majorly composed of α -synuclein and ubiquitin [1]. The development of PD is suggested to be resulting from multiple factors, but the theory of oxidative stress leading to oxidative damage [2] is evidenced by several post-mortem brain studies that showed altered brain iron content, impaired mitochondrial function, altered brain antioxidant systems such as superoxide dismutase activity and reduced glutathione levels, along with oxidative damage to lipids, proteins, and DNA [3].

Cholesterol is a component enriched in plasma membrane, growth cones, and synapses [4]. A compromised axonal plasticity is an important observation made in the cultures supplemented with cholesterol, that resulted in reduced neurite outgrowth too [5]. Adult neurons can rely on exogenous cholesterol supply for their function at a time when slight alterations only can be tolerated [4]. The cholesterol derivative, 27-hydroxycholesterol (27-OHC), increases α -synuclein levels and reduces DA synthesis by activating the liver X receptor [6, 7]. This oxidation product of cholesterol, 27-OHC (oxysterol), is shown to pass through the blood brain barrier and enters the human brain [8], demonstrating how the inaccessible plasma cholesterol affects the brain function. The effects of 24-hydroxycholesterol (24-OHC) and 27-OHC on expression levels of tyrosine hydroxylase (TH; the rate-limiting enzyme in DA synthetic pathway) on DA and noradrenaline levels and on apoptosis are shown to be involved in PD pathogenesis [7]. Increased entrance of oxysterol, the oxidized derivatives of cholesterol, into brain following hypercholesterolemia is a risk factor for neurodegeneration [9]. Increased α -synuclein aggregation, which plays a major role in PD pathogenesis, is observed when the neuronal cultures are grown in cholesterol-rich medium [10].

PD is one of the most extensively studied neurodegenerative disorders, where the parkinsonian neurotoxin, 1-methyl-4-phenylpyridinium (MPP⁺)-mediated

SH-SY5Y cellular toxicity is used as an in vitro model of the disease. MPP⁺ is actively transported into dopaminergic neurons through the plasma membrane upon accumulation by DA transporter [11]. The molecule is sequestered into mitochondria where it shows selective inhibition of complex I of the electron transport chain (ETC). This inhibition of ETC complex I interferes with ATP production, disrupts mitochondrial membrane potential, and leads to the formation of reactive oxygen species (ROS) [12]. The present study investigated the effects of exogenously supplied cholesterol into the culture medium on MPP⁺-induced cell death, the mitochondrial membrane potential, the loss mitochondrial ETC complex I, and dopaminergic deficiency using an in vitro model of PD.

Material and Methods

Human neuroblastoma cell line, SH-SY5Y, was obtained from ATCC, VA, USA. Trypsin, low glucose Dulbecco's modified Eagle's medium, fetal bovine serum, gentamicin, and tetramethylrhodamine methyl ester (TMRM) were procured from GIBCO, Invitrogen Corporation (Carlsbad, CA, USA); and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from R and D Systems (Minneapolis, MN, USA). Dimethyl sulfoxide was obtained from Merck India (Mumbai, India). Water soluble cholesterol (cholesterol with β -cyclodextrin making it water-soluble), β -cyclodextrin, MPP⁺ HCl, Tris-HCl, ethylene glycol-bis-(2-aminoethylether)-N,N,N',N' tetraacetic acid, ethylenediaminetetraacetic acid disodium salt, amphotericin-B, and Trypan blue, DA HCl, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and retinoic acid were obtained from Sigma, St. Louis, MO, USA. Absolute alcohol and isopropyl alcohol were procured from Merck, Germany. For immunocytochemistry, chicken anti-TH (Cat No.: ab76442), goat anti-microtubule associated protein-2 (MAP 2; Cat No.: sc-5359), and rabbit anti-chicken IgG horse radish peroxidase-conjugated (Cat No.: 6211458001A), and rabbit anti-goat IgG horse radish peroxidase-conjugated (Cat No.: 62114038001A) antibodies were obtained respectively from Abcam, Cambridge, UK, Santa Cruz Biotechnology, TX, USA, and Merck-Genei, Mumbai, India. Culture flasks and serological pipettes were procured from Thermo Fischer Scientific, Waltham, MA, USA. Sterilized 96-well plates, 24-well plates, 60 mm dishes, 15 and 50 mL conical tubes were obtained from Tarson Products Pvt. Ltd., West Bengal, Kolkata. Fluorescence-activated cell sorter (FACS) tubes were purchased from Corning Life Sciences, Tewksbury, MA 01876, USA.

Maintenance and Treatment Paradigm for SH-SY5Y Cells

SH-SY5Y cells maintained in low glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum; gentamicin and amphotericin B were trypsinized and passaged when reached confluency. These cells were seeded (i) in 96 well plates at a density of 1×10^5 cells/100 μ L/well, (ii) in 6-well plates at a density of 1×10^6 cells/3,000 μ L/well, (iii) in 24-well plates at a density of 1×10^5 cells/1,000 μ L/well, (iv) 60 mm dishes at a density of $1 \times$

10⁶ cells/3,000 μ L, respectively for cell viability assay, mitochondrial membrane potential by FACS analysis, TH-immunocytochemistry, and for analysis of biogenic amines by HPLC electrochemistry. To study the effect of cholesterol, these cells were grown in culture with retinoic acid (10 μ M) for 6 days for differentiation [13]. In our study, the differentiated SH-SY5Y cells were treated with water-soluble cholesterol (50 μ M) dissolved in Milli-Q water and/or MPP⁺ (1 mM) for 24 h according to the cholesterol concentration used in previous studies in human neuroblastoma cells [14]. We have calculated the molarity of cholesterol in solution based on the cholesterol content, which was 40 mg per every 1 g of cholesterol conjugated to β -cyclodextrin. β -Cyclodextrin was solubilized in type 1 Milli-Q water.

Cell Viability Assay

Following 24 h treatment, the media was aspirated off and 100 μ L of MTT reagent (1 mg/mL in growth medium) was added to the wells and incubated for 2 h in dark at 37 °C in a CO₂ incubator. The formazan formed is dissolved in dimethyl sulfoxide, 100 μ L in each well and mixed well taking care not to froth that affects the sensitivity of the assay. The cell viability experiments carried through 2 controls, wherein cell lines were exposed to either Milli-Q water or β -cyclodextrin solubilized in type 1 Milli-Q water. However, in other experiments Milli-Q type 1 water was used in the control experiments, since β -cyclodextrin control did not show any effects on cell survival. Each experiment was performed in triplicate, and absorbance was recorded at 540 nm using a microplate reader as per published protocol [15]. This experiment was repeated 4 times, in 4 different cultures at various times ($n = 4$; d.f. = 31).

Analysis of Biogenic Amines by HPLC Electrochemistry

Cells were scrapped into 50 μ L ice-cold 0.4 M HClO₄ containing ethylenediaminetetraacetic acid disodium salt, sonicated in cold under low energy, kept on ice in the dark for 30 min, and centrifuged at 12,500 rpm for 5 min, and 10 μ L of the supernatant was injected into the HPLC-ECD system for assaying biogenic amines. Before and after sample injections, a standard solution of biogenic amines containing 4 pmol of each biogenic amine, were assayed to confirm the retention time of the analytes. The flow rate was 0.7 mL/min and the detection was performed at 0.74 V with sensitivity set at 20 nA [16].

TH-Immunocytochemistry

SH-SY5Y cells (1 $\times$ 10⁵ cells/mL) were seeded on confocal dishes and differentiated by retinoic acid treatment for 6 days. After completion of the treatment period, cells were treated with MPP⁺ and/or cholesterol (50 μ M) for 24 h, fixed in 4% paraformaldehyde and processed for immunofluorescence staining of TH as described [13]. The cells were then incubated overnight at 4 °C with goat anti-MAP 2 or chicken anti-TH primary antibodies (1:250 dilution) in 0.05% Triton-X 100. The cells were washed with phosphate buffered saline (PBS) and incubated with fluorescence labelled Alexa Fluor[®] 488 donkey anti-goat IgG and Alexa Fluor[®] 568 goat anti-chicken IgG secondary antibodies (1:500 dilution) and stained with DAPI nuclear stain. Cells were examined using a confocal microscope (Andor Technology, Ireland). Fluorescence intensity of cells stained for TH-immunoreactivity (red) were measured using ImageJ software.

Mitochondrial Membrane Potential Assay by FACS

For flow cytometry, 2 $\times$ 10⁵ cells were plated for TMRM staining. Cells were trypsinized and incubated in fresh medium, containing 50 nM of TMRM, at 37 °C in CO₂ incubator for 30 min. The cells were then washed with D-PBS to remove excess of stain and resuspended in 500 μ L of D-PBS. The cells were then analyzed employing flow cytometry (BD LSR Fortessa FACS), and the software-FACS Diva 6.2 (BD Bioscience, USA). Phycoerythrin (PE-A) filter was used with an excitation and emission of 540 and 570 nm. Ten thousand of events from each sample were used for measuring the mean fluorescence intensity. The experiments were repeated 3 or more times, performed on different days.

Statistical Analysis

One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was employed for finding significant differences among different treatment groups. Values of $p \leq 0.05$ were considered statistically significant. Results are given as mean $\pm$ SEM.

Results

Cholesterol Enhanced MPP⁺-Mediated Neurotoxicity of Differentiated SH-SY5Y Neurons

The data from the β -cyclodextrin-treated control did not differ significantly when compared to type 1 Milli-Q water control in cell viability experiments. A cholesterol concentration of 50 μ M was selected to study the impact of cholesterol on MPP⁺-mediated neurotoxicity (Fig. 1a, b), and to assess the mechanisms underlying the exacerbated cell toxicity of MPP⁺ in cholesterol-treated, differentiated SH-SY5Y neurons. Differentiated SH-SY5Y cells were treated with cholesterol 50 μ M alone, MPP⁺, and both cholesterol 50 μ M and MPP⁺ for 24 h and was further subjected to cell viability assay. In the cholesterol-alone treated cells, 82% was the observed cell viability and it was observed that there was 67% cell viability in only neurotoxin-treated cells and this was decreased to 52% cell viability when cells are treated with both cholesterol 50 μ M and MPP⁺ (Fig. 1).

Effects of Cholesterol Exposure on MPP⁺-Induced Depletion in Biogenic Amines in Differentiated SH-SY5Y Neurons

Differentiated SH-SY5Y cells treated with cholesterol 50 μ M alone, MPP⁺, and both cholesterol 50 μ M and MPP⁺. These groups were analyzed for the contents of DA (Fig. 2a) and its metabolites, DOPAC and HVA. We observed that the 50% decrease in DA content in MPP⁺-treated cells was further decreased to 30% when the cells were treated with both cholesterol 50 μ M and MPP⁺ (Fig. 2a). The levels of DA metabolites, DOPAC (Fig. 2b)

Fig. 1. Cholesterol caused exacerbation of MPP⁺-induced toxicity in SH-SY5Y neurons in MTT assay. Cells were co-treated with cholesterol 50 μ M and 1 mM MPP⁺ for 24 h. **a** Phase contrast images of the neurons are depicted – Control (Con) treated with Type I Milli-Q water or β -cyclodextrin, the vehicle control (VC), cells treated Chol 50 μ M (Chol 50), or 1 mM MPP⁺ only (M), and both Chol 50 μ M and 1 mM MPP⁺ (M-Chol 50). **b** The concentration of

formazan formed as measured at absorbance 490 in each case is provided. Results given are Mean $\pm$ SEM; One-way ANOVA followed by Tukey's post-hoc test showed significant differences among different treatment groups (d.f. = 31). * $p \leq 0.05$ vs. controls (con), data were not significantly different from the β -cyclodextrin control; @ $p \leq 0.05$ vs. MPP⁺-treated group. $n = 4$ independent experiments, conducted on different days.

or HVA (Fig. 2c), were not altered significantly between the treatment groups. However, DA turnover (Fig. 2d), as depicted by the metabolites to the neurotransmitter ratio (DOPAC + HVA: DA), was significantly more in both cholesterol 50 μ M and MPP⁺-treated neurons as compared to the untreated or cholesterol-treated cells (Fig. 2d).

Imaging Differentiated SH-SY5Y Cells for Their Dopaminergic Phenotype

Neuronal maturity marker, MAP 2 immunofluorescence staining in green, indicated successful differentiation of SH-SY5Y cells in the present study (Fig. 3a, 3rd column). Co-localization as depicted by yellow/pink fluorescence staining of MAP and TH (Fig. 3a, last column) in almost every neuron in the figures suggested these differentiated neurons to be dopaminergic in nature. Cholesterol (50 μ M) or MPP⁺ (1 mM) treatment of the cells caused not only decrease in the number of dopaminergic

neurons as seen in DAPI positive cells (Fig. 3a, 4th column), but also decrease in MAP 2 (Fig. 3a, 3rd column) or TH (Fig. 3a, 2nd column) fluorescence intensity suggesting general loss of health of these neurons. A significant decrease in the fluorescence intensity of TH in differentiated SH-SY5Y cells following cholesterol or the neurotoxin, MPP⁺-treatment is found from image analysis (Fig. 3b). These independent effects were synergized when the neurons were treated with both cholesterol and MPP⁺ (Fig. 3b).

Effects of Cholesterol or MPP⁺ or Both on Mitochondrial Membrane Potential – A FACS Analysis

A cell-permeant, cationic, red-orange fluorescent dye, TMRM is readily accumulated in active mitochondria with intact membrane potential. Intact mitochondria provide a bright fluorescence signal when stained with

Fig. 2. Exacerbated loss of dopamine (DA) following combined treatment of cholesterol and MPP⁺. Cells were treated with cholesterol 50 μ M and 1 mM MPP⁺ for 24 h. Levels of DA (**a**), 3,4-dihydroxyphenylacetic acid – DOPAC (**b**), and homovanillic acid – HVA (**c**) in differentiated SH-SY5Y neurons in Milli-Q Type 1 water Control (Con) and treated with cholesterol 50 μ M (Chol 50), or 1 mM MPP⁺ (M), or with both cholesterol 50 μ M and 1 mM

MPP⁺ (M-Chol 50) are depicted. **d** Provides the ratio of (DOPAC + HVA): DA values, which represents the turnover of the neurotransmitter in the cells at the given time. Results given are Mean $\pm$ SEM; One-way ANOVA followed by Tukey's post-hoc test. The results showed significant differences in the levels of DA or its turnover only. * $p \leq 0.05$ vs. controls (con); [@] $p \leq 0.05$ vs. MPP⁺-treated group. $n = 3$ (d.f. = 11).

this potential-dependent dye. Unhealthy mitochondrial membranes fail to maintain a difference in electrical potential between the interior and exterior of the organelle, thereby ceasing accumulation of the dye within, showing dim fluorescence signal, and thereby providing a measure of mitochondrial membrane potential loss. Fluorescence intensity maxima for TMRM is with the absorbance peak at 548 nm and emission peak at 574 nm. The differentiated neurons were stained with TMRM, and analyzed in a FACS under a phycoerythrin-A. Cholesterol treatment caused about 20% loss in the mitochondrial membrane potential (Fig. 4b). MPP⁺-treated cells depicted 57% loss in the mitochondrial membrane potential of the differentiated cells, which was quite significant (ANOVA; $p \leq 0.0001$; d.f. = 11; Fig. 4b). The neurons that were treated with both cholesterol and MPP⁺ caused about 70% loss

(ANOVA; $p \leq 0.0001$; d.f. = 11; Fig. 4a, b) of the mitochondrial membrane potential as a compared to the untreated cells.

Discussion

The present study provides first-hand information on the effects of hypercholesterolemic condition on post-mitotic, mature SH-SY5Y differentiated cells, which are neurons of human origin. These cultured neurons are dopaminergic in nature, bringing the relevance of the finding for application to pathological conditions that are affected in movement and psychiatric conditions that involve cognition, emotion, and reward effects. The study brought to light extreme sensitivity of SH-SY5Y neurons

Fig. 3. Effects of cholesterol and/or MPP⁺ on tyrosine hydroxylase (TH) and microtubule associated proteins (MAP) localization and levels. **a** The differentiated cells were analysed for TH (red) and MAP (green) employing immunofluorescence cytochemistry. The cells were identified by staining the nuclei using DAPI (blue). Cells were treated with cholesterol 50 μ M and 1 mM MPP⁺ for 24 h. (Abbreviations used in the figures are as explained in Fig. 3. **b** The fluorescence intensity in cells for TH staining was evaluated as ar-

bitrary fluorescence value employing ImageJ software, and depicted as AFU per million cells. MPP⁺ and cholesterol treated group differed significantly from the MPP⁺ or cholesterol alone treated group. Results given are Mean $\pm$ SEM; One-way ANOVA followed by Tukey's post-hoc test showed significant differences from that of control values. * $p \leq 0.05$ vs. controls (con; Milli-Q Type 1 water); @ $p \leq 0.05$ vs. MPP⁺-treated group. $n = 3$, independent experiments conducted on 3 different days (d.f. = 11).

to cholesterol exposure, and their inadequacy to survive in cholesterol enriched condition. The study also revealed the heightened sensitivity of SH-SY5Y differentiated neurons to MPP⁺-neurotoxicity under hyper-cholesterol culture conditions. It is demonstrated that the hypersensitivity is in parallel with the loss of mitochondrial membrane potential loss, which in turn may result in loss of mitochondrial functions, and therefore could be the reason for the exacerbated neuronal death due to MPP⁺, and higher levels of cholesterol in the culture.

SH-SY5Y neuroblastoma cell line was selected for the present study because of its dopaminergic neuronal lineage, and its wide acceptance as an in vitro MPP⁺-induced cellular model of PD. Additionally, its ability in basal conditions to express molecules associated with lipid rafts, such as flotillin, which are proteins with predominant expression in catecholaminergic neurons adds to the merits [5, 17]. One of the major pathogenic markers of PD,

α -synuclein monomers is also shown to undergo lipid raft-dependent internalization in SH-SY5Y-glia co-cultures, heightened the relevance of the present investigation [18]. Furthermore, molecular dynamics studies evidenced binding of α -synuclein in its chimeric form, which are condensed cholesterol-ganglioside complexes found in lipid raft domains of the plasma membrane of neurons [19]. Above all, our laboratory has been using this cell line for investigating neurodegenerative disease biology for the past several years [13, 15, 20, 21].

Lipid buster statins are shown to protect against rotenone-induced neuronal death in SH-SY5Y cells, another cellular model of PD, by enhancing the upstream autophagy markers [22]. These neuroprotective effects seen in SH-SY5Y probably are through mechanisms involving SREB-2-mediated sterol synthesis, which helped to decrease lipid peroxidation thereby maintaining membrane integrity [23]. It has been shown that exposure of neuro-

Fig. 4. Cholesterol exacerbates MPP⁺-induced loss of mitochondrial membrane potential in SH-SY5Y cells. **a** Differentiated SH-SY5Y cells were stained with TMRM and analyzed in a FACS under a phycoerythrin-A filter at excitation λ 540 and emission λ 570, for the status of mitochondrial membrane potential in the Control (con; treated with Milli-Q Type 1 water) cells, and cells that were treated with either cholesterol (50 μ M; Chol 50), or 1 mM MPP⁺ (M), or with both cholesterol and MPP⁺ (M-Chol 50). **b** Slight loss

of membrane potential was evidenced following cholesterol treatment, but more than 50% loss was visible in MPP⁺ treated cells. Results given are Mean $\pm$ SEM; One-way ANOVA followed by Tukey's post-hoc test showed significant differences among different treatment groups. * $p \leq 0.05$ vs. controls (con); @ $p \leq 0.05$ vs. MPP⁺- and cholesterol-treated group (d.f. = 11). $n = 3$ independent experiments.

nal cell line to cholesterol dose-dependently increased the level of cholesterol in total cell lysates by 24 h, and exhibited no effects on neurite outgrowth or the numbers [5]. In our investigation, we have not measured the levels of cholesterol in the cells, but being under ideal and similar conditions, it is expected that there could be a significant increase in the levels of cholesterol in SH-SY5Y cells following 24 h in culture. Role of alteration in lipid raft dynamics due to cholesterol imbalance could be a mechanism involved in the neurotoxicity leading to PD and dementia with Lewy bodies [see for review; 24, 25]. It is evidenced from literature that in age-related disorders such as PD and Alzheimer's disease, changes in lipid raft composition is observed from asymptomatic stage, which becomes important with physicochemical alteration toward progression of these diseases [25]. These compelling evidences prompt the importance of cholesterol alteration in cellular model of PD as it could directly influences the neurotoxicity.

The potentiation of MPP⁺-neurotoxicity was probably due to increased membrane lipid peroxidation and free radical generation [14]. Also, cholesterol treatment reduced cell viability in SH-SY5Y cells in dose-dependent manner [26]. Literature reveals potential adverse impact of cholesterol in neurodegenerative diseases, and the fact that accumulation of non-esterified cholesterol, which predominates in CNS, regulates the cholesterol homeo-

stasis and is toxic to neuronal cells [27–30]. Accordingly, our present study demonstrated that cholesterol caused toxicity in neurons and synergised MPP⁺-neurotoxicity.

Cholesterol and the neurotoxin, MPP⁺ seem to target dopaminergic neurons as revealed by a further decrease in the intensity of TH immunofluorescence, when the neurons are treated with both cholesterol and MPP⁺. MPP⁺ causes inhibition of mitochondrial ETC complex I, resulting in loss in ATP production and an increased production of ROS, that is further enhanced by DA auto-oxidation in the midbrain area [31]. Interestingly, increased cholesterol levels cause membrane lipid peroxidation [14] and free radical generation, which adds on to the MPP⁺-neurotoxic effect to produce an exacerbated neuronal death. In general, there are 2 views on the influence of cholesterol on the progression of neurodegenerative diseases, one that supports hastening the onset of such chronic diseases; and the other that supports the notion that cholesterol may be neuroprotective [32, 33]. The dose-dependent neuroprotective effects of cholesterol on neurodegenerative diseases are probably linked to the lipid microdomains rearrangements and its binding with and translocation of toxic protein molecules (such as α -synuclein) in plasma membranes [32, 33].

The present study also reveals a reduction in the mitochondrial membrane potential due to cholesterol or the neurotoxin, and this effect is further sharpened in pres-

ence of both. MPP⁺, a strong mitochondrial complex I inhibitor affects the mitochondrial depolarization and reduces the membrane potential. There seems to be a synergistic effect of the neurotoxin and cholesterol, when present together in the culture, on the mitochondria. Cholesterol contribution may hasten the neuronal cell death since it could be metabolized as polar metabolites, toxic to the surviving neurons [34]. It is reported that the cytotoxicity in SH-SY5Y due to metabolites such as 27-OHC involves the reduction of mitochondrial membrane potential [35]. Similarly, 24-OHC also causes neurotoxicity in differentiated SH-SY5Y cells, following a decreased mitochondrial membrane potential, resulting from DNA fragmentation, caspase-3 activation, and apoptosis [36]. The report that 7-keto-cholesterol exhibited synergistic effects on MPP⁺-induced cell death in differentiated PC12 neurons in culture through decrease in the mitochondrial transmembrane potential, cytosolic accumulation of cytochrome c, activation of caspase-3, increase in the formation of ROS along with neuronal GSH depletion [37], strongly supports the present findings.

It could be concluded from the present study that high cholesterol incorporated into human neuroblastoma

cells along with MPP⁺-induced neurotoxicity worsen the cell survivability probably through the exacerbation of known mechanisms of dopaminergic cell death by the neurotoxic metabolite, MPP⁺. This apparently involves the mitochondrial ETC complex I inhibition and depolarization of membrane potential as revealed from the present investigation. Further in vivo study is warranted to understand how the neurotoxicity is exacerbated by disturbed cholesterol concentration in the brain.

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Disclosure Statement

The authors declare no conflict of interest to publish this manuscript in the *Annals of Neuroscience*.

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