CrossMark

Phytanic Acid-Induced Neurotoxicological Manifestations and Apoptosis Ameliorated by Mitochondria-Mediated Actions of Melatonin

Shaista Chaudhary[1] · Upasana Sahu[1,2] · Sudeshna Kar[2] · Suhel Parvez[1]

Abstract Phytanic acid, a saturated branched chain fatty acid and a major constituent of human diet, is predominantly found in dairy products, meat, and fish. It is a degradation product from the phytol side chain of chlorophyll. Degradation of PA is known to occur mainly in peroxisomes via α-oxidation and in mitochondria via β-oxidation. Due to its β-methyl group present at the 3-position of the carbon atoms, PA cannot be β-oxidized. Although alteration in the metabolism of PA may play an important role in neurodegeneration, the exact mechanism behind it remains to be evaluated. In this study, we have described the potential of PA to induce neurotoxicity as an in vitro model (neuronal cell line, SH-SY5Y cells). Cells were pretreated with melatonin (10 μM) for 1 h followed by with and without PA (100 μM) for 24 h. In the present study, our data has confirmed that PA markedly increased both intracellular reactive oxygen species and reactive nitrogen species levels. Our results have shown that PA treatment did not induce cell death by cleavage of caspase-3/PARP-1 mediated by mitochondria through intrinsic pathways; however, PA induced nitric oxide-dependent apoptosis in SH-SY5Y cells. Additionally, melatonin pretreatment reduced the cell death in SH-SY5Y cells. Melatonin also effectively exerted an antiapoptotic and anti-inflammatory action by regulating Bax, Bcl-2, p-NFκB, and iNOS expressions in SH-SY5Y cells. These results suggested that melatonin acted as an antioxidative and antiapoptotic agent by modulating ROS, apoptotic proteins, and inflammatory responses under BCFA-induced neurotoxic conditions. The protective effects of melatonin depend on direct scavenging activity of free radicals and indirect antioxidant effects. Further deciphering of the cellular and molecular mechanism associated with neuroprotection by melatonin is warranted in BCFA-induced neurotoxicity.

Keywords Phytanic acid · Melatonin · Oxidative stress · Apoptosis · Neurotoxicity

Introduction

Branched chain fatty acids (BCFAs) are saturated long chain molecules with methyl groups and major lipid constituents [1]. They have complex structural and functional roles in eukaryotic cells [2]. The influence of BCFA accumulation on different brain cell types in the pathogenesis of neurodegeneration is an unresolved issue [3]. It is possible that the incorporation of BCFAs might destabilize cell membranes or disturb the microenvironment of cells, which leads to dysfunction and death of vulnerable brain cells [4]. Recent evidence indicates that high concentrations of BCFAs are also involved in neuronal cell death. Some authors have shown that BCFAs are also toxic to many cell types including tumor cells [5–7].

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) (PA) is a BCFA formed by breakdown of phytol. It is present in various dietary products such as fish, meat, and in dairy products like milk, butter, and cheese [8]. Higher concentration of PA is found in tissues and body fluids of affected patients [9]. PA is degraded by α-oxidation inside the peroxisome and further through β-oxidation in mitochondria. Due to deficiency of peroxisomal enzymes which are involved in

Shaista Chaudhary and Upasana Sahu contributed equally to the study

✉ Suhel Parvez
drsuhelparvez@yahoo.com; sparvez@jamiahamdard.ac.in

[1] Department of Medical Elementology and Toxicology, Jamia Hamdard (Hamdard University), New Delhi 110062, India

[2] Jamia Hamdard Institute of Molecular Medicine, Jamia Hamdard (Hamdard University), New Delhi 110062, India

α-oxidation, PA is accumulated in various diseases with peroxisomal impairments in tissues or cells [10]. PA has a diverse range of neurotoxic effects. The mechanisms of PA-induced neurotoxicity have been investigated in several studies and has shown that PA induces production of reactive oxygen species (ROS) and multiple mitochondrial dysfunctions [11, 12]. Studies have shown that PA exerts neurotoxic effects mainly mediated by disruption of mitochondrial energy homeostasis in tissues and cultured neural cells [13, 14]. PA also induces oxidative stress in brain and heart mitochondria, generation of superoxides, mitochondrial depolarization, and cell death in neuronal cells [15–17].

Numerous studies have reported the implication of an increased generation of free radicals and oxidative stress in the neurotoxicity induction mechanism of PA [18, 19]. Many studies have demonstrated that oxidative stress is tightly associated with the pathogenesis of many diseases including various neurodegenerative diseases. Oxidative stress induces ROS such as H_2O_2 and superoxide anions, both of which induce the progression of neurodegenerative diseases, and ROS-mediated damage to biological molecules leads to apoptotic cell death [20]. Thus, suppression of ROS generation by antioxidants might be an effective strategy to protect against oxidative stress-induced cell death. Several ROS scavengers have been shown to protect against cell death or any kind of stress [21].

Melatonin is a hormone with known antioxidant properties. Melatonin and its metabolites have neuroprotective effects on cellular stress by reducing ROS and reactive nitrogen species (RNS) [22]. Several studies have reported that melatonin decreases nitric oxide by inhibition of its synthesizing nitric oxide synthase enzyme and protects the mitochondria from oxidative damage-reduced oxygen consumption, loss of membrane potential, and superoxide anion production [23–25]. In addition to these scavenging actions, melatonin provides extraordinary neuroprotection against various oxidative stress associated with different types of neurodegenerative disorders in which ROS are involved, which have an important role in inducing cell death [26]. Moreover, melatonin has been shown to reduce oxidative damage to nuclear DNA both in vivo and in vitro [27, 28]. In line with these observations, we have investigated the neurotoxic effects of PA in SH-SY5Y cells by evaluating cell viability, ROS production, apoptotic proteins, and inflammatory markers.

In the present study, we have used SH-SY5Y cells as an in vitro model to investigate the role of BCFA like PA modulation as neurotoxicity marker. SH-SY5Y cells are considered as a good cellular model for neurotoxicity evaluation since they possess many biochemical and functional properties of neurons [29]. However, to our knowledge, nothing has been reported regarding in vitro influence of PA on these parameters using SH-SY5Y cells. Although there are several reports which have investigated the therapeutic effects of melatonin on several types of peripheral and central neuropathies, there is a paucity of literature related to the neuroprotective effects of melatonin against BCFA such as PA-induced neurotoxicity. Thus, neurotoxicity have been investigated in the current study.

Materials and Methods

Chemicals

Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco BRL (Gaithersburg, MD, USA). Lactate dehydrogenase (LDH) assay kit was obtained from Cayman Chemical (Ann Arbor, MI). The antibodies of rabbit polyclonal anti-phosphorylated-NF-κB (pNF-κB), the rabbit monoclonal anti-Bax, the mouse monoclonal anti-Bcl-2, the mouse monoclonal anti-GAPDH, the mouse anti-PARP-1, the rabbit polyclonal anti-iNOS, and the rabbit anti-cleaved caspase-3 were purchased from Santa Cruz Biotechnology (Santa Cruz, Taxes, USA), and the horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and anti-rabbit IgG antibodies were purchased from Cell Signaling (Beverly, MA, USA).

Melatonin and phytanic acid were purchased from Sigma-Aldrich (St Louis, MO, USA). 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye, and Griess reagent were purchased from Sigma-Aldrich (St Louis, MO, USA). Other chemicals used in this study were obtained from Hi-Media Labs (Mumbai, India).

SH-SY5Y Cells

SH-SY5Y cell line was procured from National Centre for Cell Science (NCCS), Pune, India. SH-SY5Y cells were grown in DMEM medium containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 % heat-inactivated FBS. Cells were resuspended in DMEM with 10 % FBS and penicillin/streptomycin under a 5 % CO_2/95 % humidified air incubator. Then, the cells were seeded onto 96-well culture plates at density of 1×10^5 cells/well. All treatments were performed with 80–85 % confluent cells [30].

Treatment Regime

To investigate the neurotoxic effects of PA, cells were treated with different concentrations of PA (10, 50, 100 μM) for 24 h. Based on the dose response results, the concentration of PA (100 μM) was chosen for further experiments. In order to determine the protective effects of melatonin against PA, the next sets of experiments were performed and the SH-SY5Y cells were pretreated with melatonin (10 μM) before 1 h of PA

exposure. After adding PA, cells were further incubated for 24 h without changing the media. The control-cultured cells were incubated in culture medium. The concentrations of PA and melatonin were based on previous published studies [31, 32].

Cell Viability Assay

Cell viability was analyzed by utilizing MTT dye [33]. Briefly, 1×10^5 SH-SY5Y cells were seeded/well in a 24-well plate. After 24 h of treatment, media was replaced with serum-free DMEM media containing 0.5 mg/mL of MTT and incubated at 37 °C in a 5 % CO_2 incubator for additional of 30 min. Intracellular formazan products were solubilized by replacing MTT reagent with extraction buffer (4 mM HCl and 0.1 % NP 40 in isopropanol) and incubated for 15 min at 37 °C. Contents were transferred to a 96-well tissue culture plate, and optical density was measured at 570-nm spectral wavelength using a microplate reader (Bio-Rad, CA, USA). The results were expressed as the percentage of control.

Morphological Observation of Cells (Bright Field Imaging)

SH-SY5Y cells were seeded in 24-well plate at a density of 2×10^5 cells/well in culture medium. After the treatment, bright field images of cells were captured at ×20 magnification using NIS elements software provided with a (Nikon, Eclipse TS100, Tokyo, Japan) microscope.

LDH Assay

Cells were seeded in 96-well plate at a density of 1×10^5 cells/well in culture medium. Released lactate dehydrogenase (LDH) from damaged cells in culture medium was measured by using an LDH assay kit [34]. A total of 100 μL of culture medium was used for LDH analysis. Released LDH is catalyzed by the oxidation of lactate to pyruvate with simultaneous reduction of NAD^+ to NADH. The rate of NAD^+ reduction was measured as an increase in absorbance at 490 nm using Gen5 software provided with the microplate reader (BioTek, USA). The results were expressed as μU/mL of LDH activity in treated and untreated cells.

Measurement of ROS Level

The intracellular ROS was measured by using oxidation-sensitive fluorescent dye DCFH-DA [35]. The cells were plated at a density of 5×10^4 cells/well in 24-well plate for 24 h and treated. After treatment, cells were then incubated with 5 μM DCFH-DA for 30 min at 37 °C. The non-ionic, non-polar DCFH-DA crosses cell membranes and is hydrolyzed enzymatically by intracellular esterases to non-fluorescent

DCFH-DA which is oxidized to highly fluorescent dichlorofluorescein (DCF) in the presence of ROS. After incubation, the intracellular ROS was measured by fluorimetric detection of DCF oxidation at an excitation wavelength of 485 nm and an emission wavelength of 522 nm. The DCF fluorescence intensity is proportional to the amount of ROS formed intracellular. Images were captured using a fluorescence microscope (Nikon, Eclipse TS100, Tokyo, Japan). The DCF fluorescence-integrated density was analyzed with ImageJ software (1.50 version, NIH, USA).

NO Determination

Nitric oxide (NO) was determined by measuring the nitrite content in culture medium by using Griess reagent [36]. Briefly, the cells were seeded in 96-well plate at density of 1×10^5 cells/well and cultured overnight. At the end of treatment, culture media was centrifuged at $1000 \times g$ for 5 min at 4 °C to get rid of dead cells and cell debris. After that, 100 μL of supernatant was transferred to fresh 96-well plate and mixed with an equal volume of Griess reagent (0.04 g/mL PBS, pH 7.4) and incubated for 20 min at room temperature. Absorbance of resultant color was measured at 540 nm using Gen5 software provided with BioTek microplate reader. Concentration of nitrite in medium of the treated and untreated cells was calculated using a standard curve of sodium nitrite and expressed as percentage of control.

Western Blot Analysis for Bax, Bcl-2, Cleaved Caspase-3, PARP-1, pNF-κB, and iNOS

SH-SY5Y cells were seeded in a density of 5×10^5 cells/well in a 24-well culture plate and cultured for 24 h. After treatment, the cells were collected and rinsed twice with cold PBS and removed by scrapping and then centrifuged at $900 \times g$ for 5 min at 4 °C. Cells were lysed in a buffer containing (10 mM Tris–HCl with pH 7.4), 150 mM NaCl, 2 mM EDTA, 1 % NP-40, 0.01 % SDS protease inhibitor cocktail, and phosphatase inhibitor) directly onto the cells. Cell lysates were sonicated at 75 % energy for 2 min with 15 s on and 10 s off cycle and cleared by centrifugation at $10,000 \times g$ for 10 min at 4 °C. Supernatants were stored at 80 °C until analysis by western blot. Protein concentration was estimated according to Bradford [38] using bovine serum albumin (BSA) as standard. Equal amounts of proteins (20–40 μg/well) were resolved in 12–15 % SDS gel electrophoresis and transferred onto PVDF membranes according to conventional partially modified methods [37]. Briefly, proteins were transferred at 150 mA for 1 h to PVDF membranes using Mini Trans-Blot Cell apparatus (Bio-Rad, Hercules, CA, USA). The procedure for immunodetection includes the transfer and blocking of the membrane for 1 h at room temperature with phosphate-buffered saline-Tween (PBST) (150 mM NaCl, 10 mM KCl,

0.1 M NaH$_2$PO$_4$, 0.1 M KH$_2$PO$_4$, and 0.05 % Tween-20, pH 7.4) containing 5 % non-fat-dried milk. After that, the membranes were incubated overnight at 4 °C with primary antibodies Bax, Bcl-2, cleaved caspase-3, PARP-1, pNF-KB, and iNOS. After washing for 5–10-min periods with PBST, the membranes were incubated for 45 min at room temperature with horseradish peroxidase-conjugated secondary antibodies (1:5000 in PBST). After washing for 5–10-min periods with PBST, the detection of bound antibodies was visualized by chemiluminescence using the ECL-plus reagent. Anti-GAPDH antibody was utilized to normalize protein loading and transfer. Densitometric analysis was performed by using ImageJ software (1.50 version, NIH, USA).

Protein Estimation

The protein content was determined by the method of Bradford [38] using BSA as a standard.

Statistical Analysis

Data were expressed as mean ± S.E.M. for absolute values or percentage of control. Assays were performed in duplicate or triplicate, and all data were analyzed using analysis of variance (ANOVA) followed by Tukey's test. P values <0.05 were considered as significant. All the statistical analyses were performed using GraphPad prism 5 software (GraphPad Software, Inc. San Diego, CA, USA).

Results

Effect of PA and Melatonin on Cell Viability

To evaluate the viability of SH-SY5Y cells, MTT assay was performed after 24 h of treatment with various concentrations of PA. PA significantly decreased the viability of SH-SY5Y cells in a dose-dependent manner when compared to control cells (Fig. 1a). Thereafter, we investigated the effect of melatonin on cell viability of SH-SY5Y cells treated with PA. SH-SY5Y cells were pretreated with melatonin (10 μM) for 1 h, followed by 100 μM for 24 h. As shown in Fig. 1c, exposure to PA (100 μM) significantly reduced viability of SH-SY5Y cells when compared with untreated SH-SY5Y cells. However, pretreatment with melatonin significantly decreased PA-induced loss of cell viability. Similarly, the changes in the
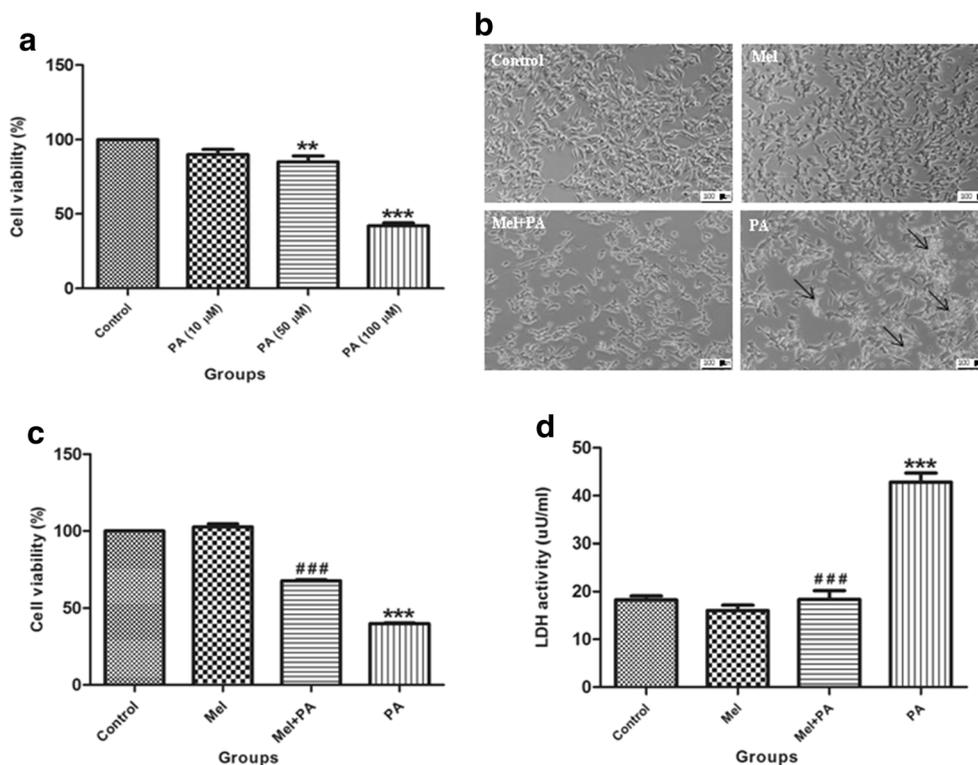

Fig. 1 Effect of different concentrations of PA on viability of SH-SY5Y cells (**a**). SH-SY5Y cells were treated with PA at 10, 50, and 100 μM for 24 h. **b** Protective effect of melatonin on PA-induced cellular morphological alteration as depicted with *black colored arrows*. SH-SY5Y cells were pretreated with 10 μM melatonin for 1 h followed by with and without 100 μM PA for 24 h. Bright field images of cells were captured at ×20 (*Scale bar* = 100 μm). **c** Effect of melatonin and PA on viability of SH-SY5Y cells. Cell viability was determined using MTT assay and presented as percentage of control. **d** Cytotoxicity was determined by LDH activity and values were expressed as μU/mL of LDH activity. The *bars* represent the mean ± S.E.M, n = 6. **P < 0.01 and ***P < 0.001 PA versus control, ###P < 0.001 Mel + PA versus PA
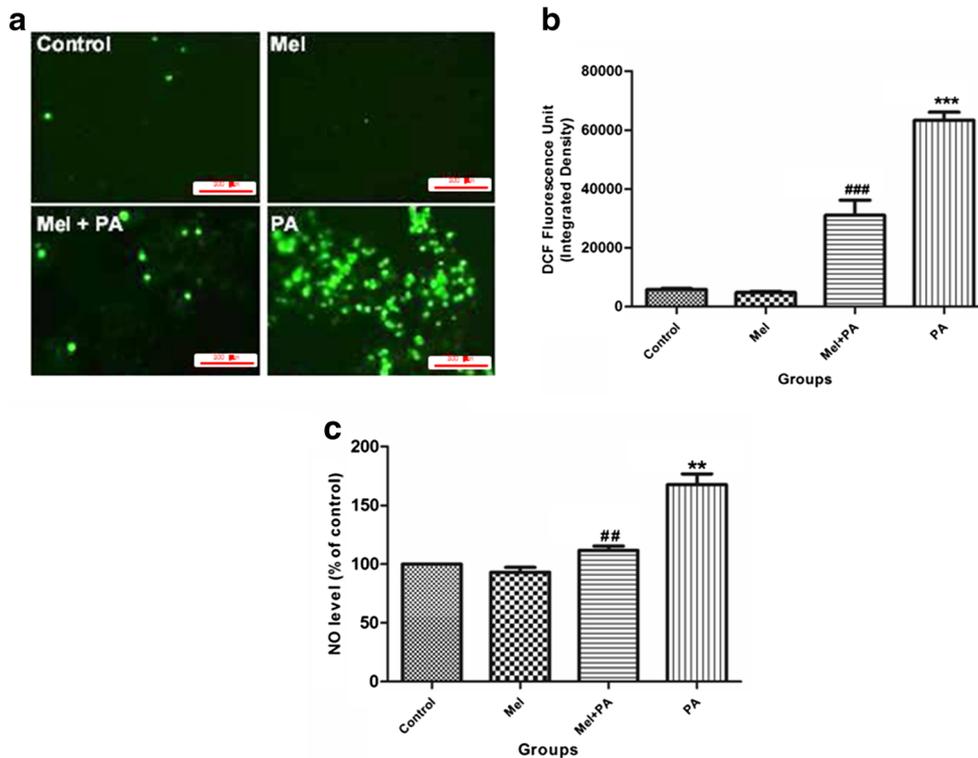
Fig. 2 Effect of melatonin and PA on ROS and NO levels in SH-SY5Y cells. ROS level was measured by DCFH-DA fluorescent dye. ROS measurement was monitored by DCF fluorescence integrated density. **a** Fluorescence microscopic images of SH-SY5Y cells demonstrating the effect of melatonin and PA. Maximum projections were generated from focus planes and images were displayed with equal pixel intensity (*Scale bar* = 100 μm). **b** The DCF fluorescence-integrated density was analyzed with ImageJ software (1.50 version, NIH, USA). The *bars* represent the mean ± S.E.M, $n = 6$. ***$P < 0.001$ PA versus control, ###$P < 0.001$ Mel + PA versus PA. **c** Effect of melatonin on PA-induced NO level. NO was determined by using griess reagent and presented as percentage of control. The *bars* represent the mean ± S.E.M, $n = 6$. **$P < 0.01$ PA versus control, ##$P < 0.01$ Mel + PA versus PA

morphology of cells are the important indicators of cytotoxicity. Untreated control cells showed normal appearance of SH-SY5Y, which were elongated and flattened with axon-like outgrowths, while after exposure to PA for 24 h, SHSY5Y cells were shrunken, rounded, and condensed and exhibited altered morphology with more detached cells when compared with control group cells as shown in bright field images (Fig. 1b).

Fig. 3 Effect of melatonin and PA on **a** Bax and **b** Bcl-2 protein expressions in SH-SY5Y cells. SH-SY5Y cells were pretreated with 10 μM melatonin for 1 h followed by with and without 100 μM PA for 24 h. Representative western blots are showing levels of Bax and Bcl-2 in SH-SY5Y cells. The relative intensities of Bax and Bcl-2 protein bands were analyzed and normalized to GAPDH. The *bars* represent the mean ± S.E.M, $n = 3$. *$P < 0.05$ and **$P < 0.01$ PA versus control, #$P < 0.05$ Mel + PA versus PA
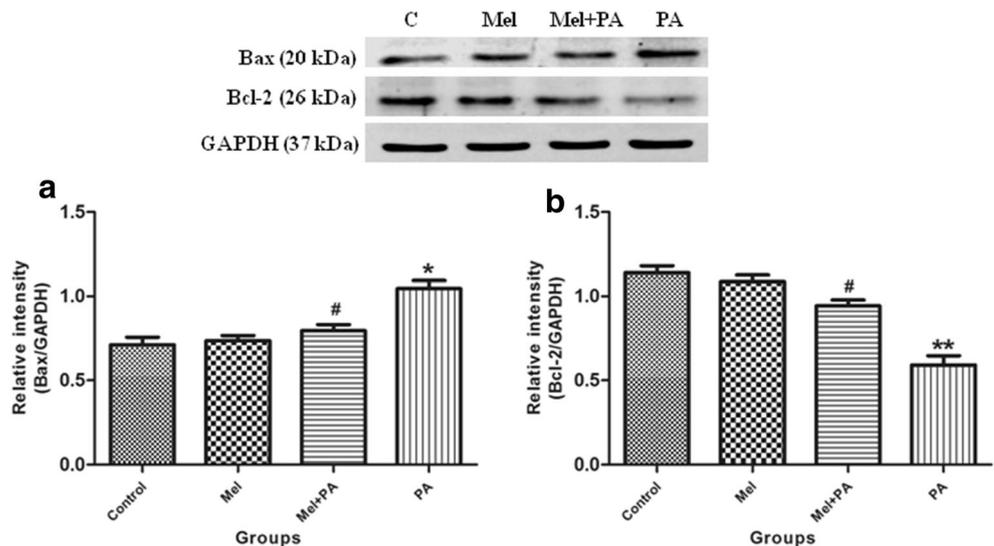
Fig. 4 Effect of melatonin and PA on **a** cleaved caspase-3 and **b** PARP-1 protein expressions in SH-SY5Y cells. SH-SY5Y cells were pretreated with 10 μM melatonin for 1 h followed by with and without 100 μM PA for 24 h. Representative western blots are showing levels of cleaved caspase-3 and PARP-1 in SH-SY5Y cells. The relative intensities of cleaved caspase-3 and PARP-1 proteins bands were analyzed and normalized to GAPDH. The *bars* represent the mean ± S.E.M, *n* = 3. No significant differences were observed
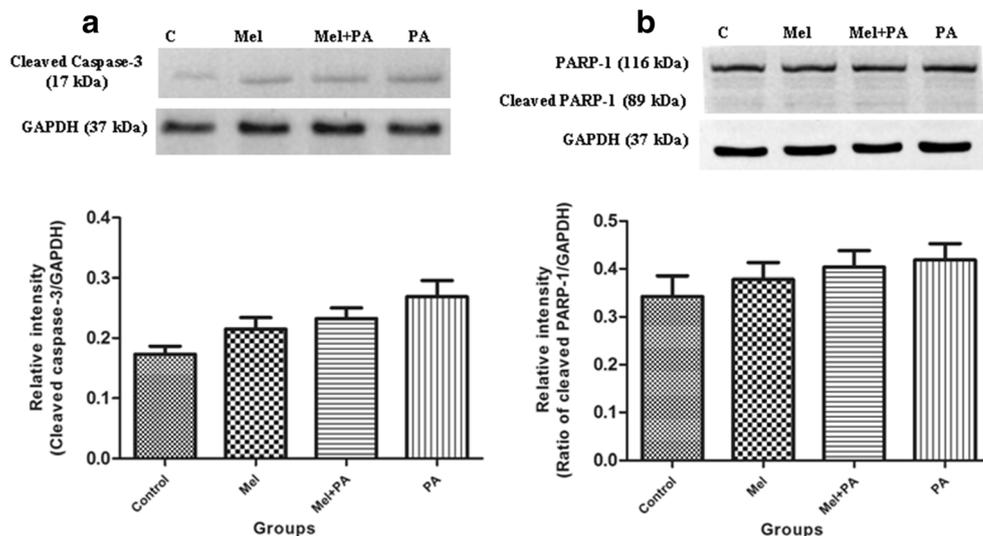
Effect of PA and Melatonin on the Activity of LDH

Cytotoxicity of PA was also confirmed by LDH leakage assay. The activity of intracellular LDH was enhanced with increase in the number of dead cells. A significant enhancement was observed in the activity of LDH in PA-treated cell culture supernatant when compared to control cells (Fig. 1d). Pretreatment of melatonin was significantly able to reverse the activity of LDH induced by PA when compared with PA-treated cells. Melatonin alone pretreated cells did not show any significant alteration in the activity of LDH when compared to control cells.

Effect of PA and Melatonin on ROS and NO

To determine whether PA treatment caused oxidative stress, the effects of PA on ROS generation was measured in SH-SY5Y cells by using DCFH-DA fluorescent-labeled probe/dye. Figure 2a shows the production of ROS in SH-SY5Y cells with altered morphology with increasing fluorescence. Images were obtained from three independent experiments. We found that PA treatment showed significantly higher level of green fluorescence density when compared with control cells (Fig. 2b). Melatonin pretreatment significantly mitigated the level of ROS production induced by PA exposure when compared to the PA-treated cells. Pretreatment of cells with melatonin did not show any significant changes when compared to control cells. Figure 2c shows significant increase in the level of NO in PA-treated SH-SY5Y cells as compared to control cells. Pretreatment of melatonin significantly decreased the enhanced level of NO induced by PA as compared with PA-treated cells. Melatonin alone treatment caused no significant change on the level of NO as compared to control.
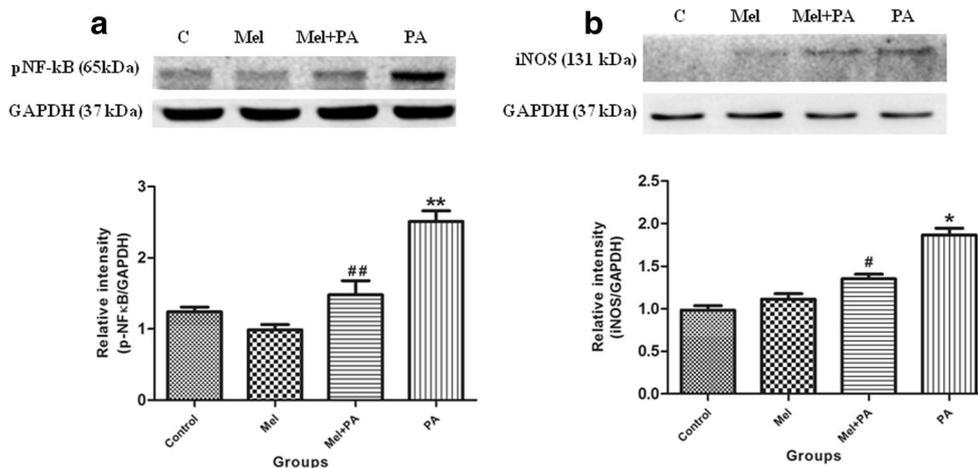

Fig. 5 Effect of melatonin and PA on **a** pNF-κB and iNOS proteins expressions in SH-SY5Y. SH-SY5Y cells were pretreated with 10 μM melatonin for 1 h followed by with and without 100 μM PA for 24 h. Representative western blots are showing levels of pNF-κB and iNOS in SH-SY5Y cells. The relative intensities of pNF-κB and iNOS protein bands were analyzed and normalized to GAPDH. The *bars* represent the mean ± S.E.M, *n* = 3. *$P < 0.05$ and **$P < 0.01$ PA versus control, #$P < 0.05$ and ##$P < 0.01$ Mel + PA versus PA

Effect of PA and Melatonin on Apoptotic Proteins

Our results show that PA (Fig. 3a) significantly elevated the expression of proapoptotic Bax protein and significantly diminished the expression of the antiapoptotic Bcl-2 protein (Fig. 3b) when compared with control SH-SY5Y cells. Interestingly, melatonin pretreatment significantly replenished the expression of Bax and Bcl-2 protein when compared to PA-treated SH-SY5Y cells. Melatonin only exposure led to no significant change in expression of proapoptotic and antiapoptotic proteins as compared to control group. The protein bands were quantified using densitometry and their differences are represented in the graph. Figure 4a, b indicates the effects of PA on the activities of cleaved caspase-3 and PARP-1. PA did not show any significant changes in the expressions of cleaved caspase-3 and PARP-1 when compared to control cells. Melatonin, preexposure has shown no significant modulations in the expressions of cleaved caspase-3 and PARP-1 when compared with the PA-treated cells.

Effect of PA and Melatonin on pNF-κB and iNOS

Figure 5 indicates the expressions of (a) pNF-κB and (b) iNOS proteins. A significant increase was observed in the expressions of pNF-κB and iNOS in PA exposed cells when compared with control cells. The enhanced levels of pNF-κB and iNOS were restored significantly by the pretreatment of SH-SY5Y with melatonin as compared to the PA-exposed cells.

Discussion

The excess accumulation of PA is a characteristic of fatty acid oxidation defects in peroxisomes, particularly neurological disorders induced by the PA toxicity [39]; however, the exact mechanisms underlying the neurotoxicity of PA remain to be elucidated. The results of the present study describe numerous findings in which we have observed the neurotoxic effects of PA on cell viability, oxidative stress, apoptosis, and neuroinflammation and their restoration by the pretreatment of melatonin in SH-SY5Y cells. Initially, we observed that the viability of SH-SY5Y cells was decreased in a dose-dependent manner by the induction of PA, which shows that the higher the PA concentrations, the greater the reduction in cell viability. The protective role of melatonin on PA-induced cell death in SH-SY5Y has also been emphasized with morphological alterations. In the present study, we found that pretreatment of melatonin was able to protect PA-induced reduction in viability of SH-SY5Y cells. A prior study also demonstrated that PA reduces cell viability in rat hippocampal astrocytes [15]. The protective effect of melatonin was further confirmed by the LDH assay. The assay is based on the principle that there is an increase in the leakage of cytosolic LDH with increasing

number of dead cells [40]. The release of LDH reflects loss of plasma membrane integrity, and extracellular LDH activity has been used for decades as a simple and reliable indicator of cell death [41]. In the present study, treatment with PA induced elevation in the LDH leakage in the medium of SH-SY5Y. Pretreatment of SH-SY5Y cells with melatonin showed lower LDH release compared to the PA-exposed cell group demonstrating its protective effect against PA-induced cytotoxicity.

Oxidative stress has been implicated in the pathophysiology of neurodegenerative diseases. Thus, the ability to determine ROS can provide important clues about the physiological status of the cell [42]. In our study, we observed the effects of PA on the level of ROS production. The level of ROS production was enhanced by the treatment of PA in SH-SY5Y cells. Increased ROS production can overwhelm endogenous antioxidant defenses and attack biological macromolecules such as lipids, proteins, and DNA leading to cell death [43]. Pretreatment of melatonin significantly attenuated increased production of ROS induced by PA in SHSY5Y cells. Inhibition of ROS production by melatonin may be an effective strategy to ameliorate against oxidative stress-induced cell death [44]. In our study, it has been proposed that PA can exert neuronal toxicity through higher production of not only ROS but also reactive nitrogen species (RNS) such as NO and peroxynitrite which also promotes oxidative stress.

In addition, we studied the effects of PA on the level of NO by measuring its oxidation product. NO is one of the highly reactive free radical which causes cell damage due to higher production of RNS. NO is produced from L-arginine by the enzyme nitric oxide synthase (NOS) and forms peroxynitrite anion by reacting with oxygen radicals and can easily oxidize proteins, lipids, and DNA [45]. In the present study, we observed that PA induction caused a significant increase in the level of NO in SH-SY5Y cells, which was effectively restored by the pretreatment of melatonin, thereby suggesting that antioxidative effects of melatonin directly contribute to scavenge peroxynitrite [46]. Various studies have also demonstrated that melatonin decreases NO by inhibition of its synthesizing NOS enzyme [23, 47]. Elevated level of NO can therefore impair the mitochondrial respiratory chain and eventually lead to apoptosis in cells [48]. In our study, PA-induced neurotoxicity might be associated with oxidative stress-induced mitochondrial dysfunction which contributed toward neuronal cell death, as many apoptotic molecules are released from the mitochondria during the apoptotic process [49]. Additionally, our study also highlighted the effects of PA exposure on antiapoptotic and proapoptotic proteins in SH-SY5Y cells.

The Bcl-2 family proteins such as Bax and Bcl-2 are important regulators of apoptosis through mitochondrial pathways [50]. The proapoptotic protein Bax destabilizes mitochondrial membrane potential and facilitates cytochrome-c release while the antiapoptotic protein Bcl-2 stabilizes the mitochondrial membrane barrier and inhibits the cytochrome c

release [51]. In our findings, we found that PA treatment caused an induction in the expression of Bax protein and inhibition in the expression of Bcl-2 protein. Further, supplementation of melatonin prior to PA, restored the levels of these proteins in SH-SY5Y cells which indicate that melatonin has shown antiapoptotic effect on the neuronal cells and consequently reduced the apoptosis rate induced by PA treatment.

Next, the signaling proteins of mitochondrial cell death cascades were studied to determine key molecules involved in PA-induced neuronal cell death in SH-SY5Y cells. Caspases are a family of proteins that execute terminal steps of apoptosis and regulate upstream induction of cell destruction [52]. Among the caspases, caspase-3 plays an important role in mitochondrial dysfunction after the release of cytochrome-c. Activated caspase-3 catalyzes intracellular protein degradation, including that of PARP-1. PARP-1 is an intracellular nuclear protein important for DNA repair, cell proliferation, and apoptosis [53]. However, in our study, PA treatment failed to induce activation of cleaved caspase-3 and fragmentation of PARP-1 in SH-SY5Y cells. These findings of our study were similar to previous report in which it has been shown that BCFA did not induce apoptosis via caspase cascade [4]. Conclusively, in our study, activation of caspase cascade was not responsible for the programmed cell death caused by BCFA such as PA in SH-SY5Y.

To further determine the involvement of PA in inflammatory reactions, we also examined the inflammatory action of PA on the expression of NF-κB in SH-SY5Y cells. NF-κB is an important transcription factor which plays a prominent role in stress, immune responses, inflammation, and apoptosis [54]. In neuroinflammatory process, redox-sensitive transcription factor such as NF-κB may be initiated by the increased ROS production or through the activation of inflammatory cytokines [55]. Treatment of SH-SY5Y cells with PA caused an increase phosphorylation of NF-κB. On the other hand, melatonin treatment has shown anti-inflammatory action by reducing the increased activation of NF-κB or production of inflammatory cytokine in SH-SY5Y cells. In several studies, it has been demonstrated that NF-κB activation is associated with upregulation of NOS enzyme activity and induction of apoptosis [56]. To further corroborate this study, we investigated whether or not PA was capable of inducing apoptosis mediated by iNOS, because in our study, PA treatment was not able to cause apoptosis through caspase-dependent pathway in SH-SY5Y cells. Induction of iNOS expression was increased as a consequence of PA treatment in SH-SY5Y cells. The induction of iNOS was obliterated by the pretreatment of melatonin to the cells. Further, we were able to show that stimulation of iNOS expression is a necessary step during PA-induced cell death. In support of our results, a previous study has also shown that PA induced apoptosis in vascular smooth muscle cells through upregulation of iNOS [4]. So far, it is not entirely clear, however, whether or not iNOS induction was sufficient to induce apoptosis in SH-SY5Y cells. We can speculate

that the production of ROS and RNS, alteration in apoptotic proteins, and activation of NF-κB expression may also be involved in PA-induced cell death. Several groups have also observed that the expression of iNOS gene is tightly regulated by NF-κB with several responsive elements being found in the promoter region of the iNOS gene [57, 58]. Accordingly, we have observed a significant activation of NF-κB in SH-SY5Y neuronal cells. We postulate that the beneficial effects of melatonin seen in our study may be mediated by similar mechanisms, thereby ameliorating PA-induced neurotoxicity, which in turn results in the protection against neuronal dysfunction in SH-SY5Y cells induced by PA.

In the present study, we investigated the potential neurotoxic effects of BCFA such as PA in SH-SY5Y cells as an in vitro model. Taking an overview of the results obtained from our study and prior findings reported by similar studies, we can conclude that PA has various mechanisms to induce neurotoxicity such as by oxidative stress, cell death, and inflammation. The findings of the present study suggest that PA treatment induces oxidative damage in SH-SY5Y cells objectified by a decrease in cell viability and higher production of ROS and RNS. In the present study, we also confirmed that treatment of PA induced cell death by the altering apoptotic proteins. However, we found that melatonin pretreatment controlled cell death. Our results clearly demonstrated that melatonin has the propensity to ameliorate PA-induced neurotoxicity by enhancing antioxidant capacity, thereby attenuating free radical-induced damage. In addition, melatonin has shown itself to have antioxidative, antiapoptotic, and anti-inflammatory properties by modulating oxidative stress, apoptotic proteins, and mitigating inflammatory responses under PA-induced neurotoxic conditions.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Ran-Ressler RR, Devapatla S, Lawrence P, Brenna JT (2008) Branched chain fatty acids are constituents of the normal healthy newborn gastrointestinal tract. Pediatr Res 64:605–609

2. Ran-Ressler RR, Sim D, O'Donnell-Megaro AM, Bauman DE, Barbano DM, Brenna JT (2011) Branched chain fatty acid content of United States retail cow's milk and implications for dietary intake. Lipids 46:569–576

3. Ferdinandusse S, Rusch H, Van Lint AEM, Dacremont G, Wanders RJA, Vreken P (2002) Stereochemistry of the peroxisomal branched-chain fatty acid alpha- and beta-oxidation systems in

patients suffering from different peroxisomal disorders. J Lipid Res 43:438–444

4. Idel S, Ellinghaus P, Wolfrum C, Nofer JR, Gloerich J, Assmann G, Spener F, Seedorf U (2002) Branched chain fatty acids induce nitric oxide-dependent apoptosis in vascular smooth muscle cells. J Biol Chem 277:49319–49325

5. Kruska N, Reiser G (2011) Phytanic acid and pristanic acid, branched-chain fatty acids associated with Refsum disease and other inherited peroxisomal disorders, mediate intracellular Ca^{2+} signaling through activation of free fatty acid receptor GPR40. Neurobiol Dis 43:465–472

6. Singh VK, Hattangady DS, Giotis ES, Singh AK, Chamberlain NR, Stuart MK, Wilkinson BJ (2008) Insertional inactivation of branched-chain alpha-keto acid dehydrogenase in *Staphylococcus aureus* leads to decreased branched-chain membrane fatty acid content and increased susceptibility to certain stresses. Appl Environ Microbiol 74:5882–5890

7. Wongtangtintharn S, Oku H, Iwasaki H, Toda T (2004) Effect of branched-chain fatty acids on fatty acid biosynthesis of human breast cancer cells. J Nutr Sci Vitaminol 50:137–143

8. Hellgren LI (2010) Phytanic acid—an overlooked bioactive fatty acid in dairy fat? Ann N Y Acad Sci 1190:42–49

9. Yepuri NR, Holt SA, Moraes G, Holden PJ, Hossain KR, Valenzuela SM, James M, Darwish TA (2014) Stereoselective synthesis of perdeuterated phytanic acid, its phospholipid derivatives and their formation into lipid model membranes for neutron reflectivity studies. Chem Phys Lipids 183:22–33

10. Kataria Y, Wright M, Deaton RJ, Rueter EE, Rybicki BA, Moser AB, Ananthanrayanan V, Gann PH (2015) Dietary influences on tissue concentrations of phytanic acid and AMACR expression in the benign human prostate. Prostate 75:200–210

11. Borges CG, Canani CR, Fernandes CG, Zanatta Â, Seminotti B, Ribeiro CAJ, Leipnitz G, Vargas CR et al (2015) Reactive nitrogen species mediate oxidative stress and astrogliosis provoked by in vivo administration of phytanic acid in cerebellum of adolescent rats: a potential contributing pathomechanism of cerebellar injury in peroxisomal disorders. Neuroscience 304:122–132

12. Busanello ENB, Amaral AU, Tonin AM, Zanatta Â, Viegas CM, Vargas CR, Wajner M (2012) Disruption of mitochondrial homeostasis by phytanic acid in cerebellum of young rats. Cerebellum 12:362–369

13. Leipnitz G, Amaral AU, Zanatta A, Seminotti B, Fernandes CG, Knebel LA, Vargas CR, Wajner M (2010) Neurochemical evidence that phytanic acid induces oxidative damage and reduces the antioxidant defenses in cerebellum and cerebral cortex of rats. Life Sci 87:275–280

14. Rönicke S, Kruska N, Kahlert S, Reiser G (2009) The influence of the branched-chain fatty acids pristanic acid and Refsum disease-associated phytanic acid on mitochondrial functions and calcium regulation of hippocampal neurons, astrocytes, and oligodendrocytes. Neurobiol Dis 36:401–410

15. Kahlert S, Schonfeld P, Reiser G (2005) The Refsum disease marker phytanic acid, a branched chain fatty acid, affects Ca^{2+} homeostasis and mitochondria, and reduces cell viability in rat hippocampal astrocytes. Neurobiol Dis 18:110–118

16. Reiser G, Schönfeld P, Kahlert S (2006) Mechanism of toxicity of the branched-chain fatty acid phytanic acid, a marker of Refsum disease, in astrocytes involves mitochondrial impairment. Int J Dev Neurosci 24:113–122

17. Grings M, Tonin AM, Knebel LA, Zanatta A, Moura AP, Filho CSD, Wajner M, Leipnitz G (2012) Phytanic acid disturbs mitochondrial homeostasis in heart of young rats: a possible pathomechanism of cardiomyopathy in Refsum disease. Mol Cell Biochem 366:335–343

18. Schönfeld P, Reiser G (2006) Rotenone-like action of the branched-chain phytanic acid induces oxidative stress in mitochondria. J Biol Chem 281:7136–7142

19. Komen JC, Distelmaier F, Koopman WJH, Wanders RJA, Smeitink J, Willems PHMG (2007) Phytanic acid impairs mitochondrial respiration through protonophoric action. Cell Mol Life Sci 64:3271–3281

20. Jenner P (2003) Oxidative stress in Parkinson's disease. Ann Neurol 53:S26–S36

21. Glade MJ, Smith K, Meguid MMA (2015) A glance at … nutritional antioxidants and testosterone secretion. Nutrition 31:1295–1298

22. Zhang HM, Zhang Y (2014) Melatonin: a well-documented antioxidant with conditional pro-oxidant actions. J Pineal Res 57:131–146

23. Kantar S, Türközkan N, Bircan FS, Paşaoğlu ÖT (2015) Beneficial effects of melatonin on serum nitric oxide, homocysteine, and ADMA levels in fructose-fed rats. Pharm Biol 53:1035–1041

24. Waseem M, Tabassum H, Parvez S (2016) Neuroprotective effects of melatonin as evidenced by abrogation of oxaliplatin induced behavioral alterations, mitochondrial dysfunction and neurotoxicity in rat brain. Mitochondrion 30:168–176

25. López A, García JA, Escames G, Venegas C, Ortiz F, López LC, Acuña-Castroviejo D (2009) Melatonin protects the mitochondria from oxidative damage reducing oxygen consumption, membrane potential, and superoxide anion production. J Pineal Res 46:188–198

26. Chen J, Wang L, Wu C, Hu Q, Gu C, Yan F, Li J, Yan W et al (2014) Melatonin-enhanced autophagy protects against neural apoptosis via a mitochondrial pathway in early brain injury following a subarachnoid hemorrhage. J Pineal Res 56:12–19

27. Karbownik M, Tan DX, Reiter RJ (2000) Melatonin reduces the oxidation of nuclear DNA and membrane lipids induced by the carcinogen delta-aminolevulinic acid. Int J Cancer 88:7–11

28. Pan X, Zhu L, Lu H, Wang D, Lu Q, Yan H (2015) Melatonin attenuates oxidative damage induced by acrylamide in vitro and in vivo. Oxidative Med Cell Longev 703709

29. Huang S, He H, Zou K, Bai C, Xue Y, Wang J, Chen J (2014) Protective effect of tomatine against hydrogen peroxide-induced neurotoxicity in neuroblastoma (SH-SY5Y) cells. J Pharm Pharmacol 66:844–854

30. Wisessmith W, Phansuwan-Pujito P, Govitrapong P, Chetsawang B (2009) Melatonin reduces induction of Bax, caspase and cell death in methamphetamine-treated human neuroblastoma SH-SY5Y cultured cells. J Pineal Res 46:433–440

31. Schönfeld P, Kahlert S, Reiser G (2006) A study of the cytotoxicity of branched-chain phytanic acid with mitochondria and rat brain astrocytes. Exp Gerontol 41:688–696

32. Moravčík R, Okuliarová M, Kováčová E, Zeman M (2014) Diquat-induced cytotoxicity on Vero and HeLa cell lines: effect of melatonin and dihydromelatonin. Interdiscip Toxicol 7:184–188

33. Yew MY, Koh RY, Chye SM, Othman I, Ngi KY (2014) Edible bird's nest ameliorates oxidative stress-induced apoptosis in SH-SY5Y human neuroblastoma cells. BMC Complement Altern Med 13:391

34. Suematsu N, Hosoda M, Fujimori K (2011) Protective effects of quercetin against hydrogen peroxide-induced apoptosis in human neuronal SH-SY5Y cells. Neurosci Lett 504:223–227

35. Venkataramana M, Chandra Nayaka S, Anand T, Rajesh R, Aiyaz M, Divakara ST, Murali HS, Prakash HS et al (2014) Zearalenone induced toxicity in SHSY-5Y cells: the role of oxidative stress evidenced by N-acetyl cysteine. Food Chem Toxicol 65:335–342

36. Czapski GA, Sun GY, Strosznajder JB (2002) Inhibition of N-methyl-D-aspartic acid-nitric oxide synthase in rat hippocampal slices by ethanol: evidence for the involvement of tetrahydrobiopterin but not lipid peroxidation. J Biomed Sci 9:3–9

37. Sahu U, Sidhar H, Ghate PS, Advirao GM, Raghavan SC, Giri RK (2013) A novel anticancer agent, 8-Methoxypyrimido[4′,5′:4,5]thieno(2,3-b) Quinoline-4(3H)-one induces neuro 2a neuroblastoma cell death through p53-dependent, caspase dependent and -independent apoptotic pathways. PLoS One 8:e66430

38. Bradford M (1976) Rapid and sensitive method for quantification of microgram quantities of protein utilizing principle of protein-dye-binding. Anal Biochem 72:248–254

39. Schönfeld P, Kahlert S, Reiser G (2004) In brain mitochondria the branched-chain fatty acid phytanic acid impairs energy transduction and sensitizes for permeability transition. Biochem J 383:121–128

40. Nirmaladevi D, Venkataramana M, Chandranayaka S, Ramesha A, Jameel NM, Srinivas C (2014) Neuroprotective effects of bikaverin on H_2O_2-induced oxidative stress mediated neuronal damage in SH-SY5Y cell line. Cell Mol Neurobiol 34:973–985

41. Abarikwu SO, Farombi EO (2015) Atrazine induces apoptosis of SH-SY5Y human neuroblastoma cells via the regulation of Bax/Bcl-2 ratio and caspase-3-dependent pathway. Pestic Biochem Physiol 118:90–98

42. Martins JB, Bastos ML, Carvalho F, Capela JP (2013) Differential effects of methyl-4-phenylpyridinium ion, rotenone, and paraquat on differentiated SH-SY5Y cells. J Toxicol 2013:1–10

43. Yi F, He X, Wang D (2013) Lycopene protects against MPP^+-induced cytotoxicity by maintaining mitochondrial function in SH-SY5Y cells. Neurochem Res 38:1747–1757

44. Tosun M, Soysal Y, Mas NG, Karabekir HS (2015) Comparison of the effects of 13-cis retinoic acid and melatonin on the viabilities of SH-SY5Y neuroblastoma cell line. J Korean Neurosurg Soc 57:147–151

45. Deniz E, Colakoglu N, Sari A, Sonmez MF, Tugrul I, Oktar S, İlhan S, Sahna E (2006) Melatonin attenuates renal ischemia–reperfusion injury in nitric oxide synthase inhibited rats. Acta Histochem 108:303–309

46. Titze-de-Almeida SS, Lustosa CF, Horst CH, Bel E, Del R, Titze-de-Almeida R (2014) Interferon gamma potentiates the injury caused by $MPP^{(+)}$ on SH-SY5Y cells, which is attenuated by the nitric oxide synthases inhibition. Neurochem Res 39:2452–2464

47. Song J, Kang SM, Lee KM, Lee JE (2015) The protective effect of melatonin on neural stem cell against LPS-induced inflammation. Biomed Res Int 854359

48. Garcimartín A, Merino JJ, González M, Sánchez-Reus M, Sánchez-Muniz FJ, Bastida S, Benedí J (2014) Organic silicon protects human neuroblastoma SH-SY5Y cells against hydrogen peroxide effects. BMC Complement Altern Med 14:384

49. Wang XJ, Xu JX (2005) Salvianic acid a protects human neuroblastoma SH-SY5Y cells against MPP^+-induced cytotoxicity. Neurosci Res 51:129–138

50. Abarikwu SO (2014) Protective effect of quercetin on atrazine-induced oxidative stress in the liver, kidney, brain, and heart of adult Wistar rats. Toxicol Int 21:148–155

51. Abarikwu SO, Farombi EO, Pant AB (2011) Biflavanone-kolaviron protects human dopaminergic SH-SY5Y cells against atrazine induced toxic insult. Toxicol Vitr 25:848–858

52. Bavithra S, Selvakumar K, Krishnamoorthy G, Venkataraman P, Arunakaran J (2013) Melatonin attenuates polychlorinated biphenyls induced apoptosis in the neuronal cells of cerebral cortex and cerebellum of adult male rats-in vivo. Environ Toxicol Pharmacol 36:152–163

53. Kauppinen T, Mauppinen TM (2007) Multiple roles for poly(ADP-ribose)polymerase-1 in neurological disease. Neurochem Int 50:954–958

54. Abdelsalam RM, Safar MM (2015) Neuroprotective effects of vildagliptin in rat rotenone Parkinson's disease model: role of RAGE-NFκB and Nrf2-antioxidant signaling pathways. J Neurochem 133:700–707

55. Kermanian F, Soleimani M, Pourheydar B, Samzadeh-Kermani A, Mohammadzadeh F, Mehdizadeh M (2014) Effects of adenosine A2a receptor agonist and antagonist on cerebellar nuclear factor-kB expression preceded by MDMA toxicity. Med J Islam Repub Iran 28:120

56. Permpoonputtana K, Govitrapong P (2012) The anti-inflammatory effect of melatonin on methamphetamine-induced proinflammatory mediators in human neuroblastoma dopamine SH-SY5Y cell lines. Neurotox Res 23:189–199

57. Song JX, Shaw PC, Sze CW, Tong Y, Yao XS, Ngi TB, Zhang YB (2010) Chrysotoxine, a novel bibenzyl compound, inhibits 6-hydroxydopamine induced apoptosis in SH-SY5Y cells via mitochondria protection and NF-κB modulation. Neurochem Int 57:676–689

58. Abbasi Habashi S, Sabouni F, Moghimi A, Ansari Majd S (2016) Modulation of lipopolysaccharide stimulated nuclear factor kappa B mediated iNOS/NO production by bromelain in rat primary microglial cells. Iran Biomed J 20:33–40