Metabolic Brain Disease
https://doi.org/10.1007/s11011-020-00559-6

ORIGINAL ARTICLE

# Neuroprotective effects of boron nitride nanoparticles in the experimental Parkinson's disease model against MPP+ induced apoptosis

Recep Küçükdoğru[1] · Hasan Türkez[1] · Mehmet Enes Arslan[1] · Özlem Özdemir Tozlu[1] · Erdal Sönmez[2] · Adil Mardinoğlu[3,4] · Ivana Cacciatore[5] · Antonio Di Stefano[5]

Received: 11 October 2019 / Accepted: 5 March 2020


## Abstract

Parkinson's disease (PD) is one of the most aggressive neurodegenerative diseases and characterized by the loss of dopamine-sensitive neurons in the substantia nigra region of the brain. There is no any definitive treatment to completely cure PD and existing treatments can only ease the symptoms of the disease. Boron nitride nanoparticles have been extensively studied in nano-biological studies and researches showed that it can be a promising candidate for PD treatment with its biologically active unique properties. In the present study, it was aimed to investigate ameliorative effects of hexagonal boron nitride nanoparticles (hBNs) against toxicity of 1-methyl-4-phenylpyridinium ($MPP^+$) in experimental PD model. Experimental PD model was constituted by application of $MPP^+$ to differentiated pluripotent human embryonal carcinoma cell (Ntera-2, NT-2) culture in wide range of concentrations (0.62 to 2 mM). Neuroprotective activity of hBNs against $MPP^+$ toxicity was determined by cell viability assays including MTT and LDH release. Oxidative alterations by hBNs application in PD cell culture model were investigated using total antioxidant capacity (TAC) and total oxidant status (TOS) tests. The impacts of hBNs and $MPP^+$ on nuclear integrity were analyzed by Hoechst 33258 fluorescent staining method. Acetylcholinesterase (AChE) enzyme activities were determined by a colorimetric assay towards to hBNs treatment. Cell death mechanisms caused by hBNs and MPP+ exposure was investigated by flow cytometry analysis. Experimental results showed that application of hBNs increased cell viability in PD model against $MPP^+$ application. TAS and TOS analysis were determined that antioxidant capacity elevated after hBNs applications while oxidant levels were reduced. Furthermore, flow cytometric analysis executed that $MPP^+$ induced apoptosis was prevented significantly ($p < 0.05$) after application with hBNs. In a conclusion, the obtained results indicated that hBNs have a huge potential against $MPP^+$ toxicity and can be used in PD treatment as novel neuroprotective agent and drug delivery system.

**Keywords** Parkinson's disease · 1-methyl-4-phenylpyridinium · Hexagonal boron nitride nanoparticles · In vitro · Neuroprotective agent · Experimental Parkinson's disease model

✉ Mehmet Enes Arslan
enes.aslan@erzurum.edu.tr

[1] Department of Medical Biology, Faculty of Medicine, Atatürk University, Erzurum 25240, Türkiye

[2] Department of Physics, Kazım Karabekir Education Faculty, Atatürk University, Erzurum, Turkey

[3] Centre for Host-Microbiome Interactions, Faculty of Dentistry, Oral & Craniofacial Sciences, King's College London, London SE1 9RT, UK

[4] Science for Life Laboratory, KTH - Royal Institute of Technology, SE-17121 Stockholm, Sweden

[5] Department of Pharmacology, G. D'Annunzio University, Chieti, Italy

## Introduction

Parkinson's disease (PD) is defined as one of the most common degenerative neurological diseases with motor and non-motor symptoms. It is characterized pathologically by the loss of neurons in the substantia nigra (SN) region where is a basal ganglia structure located in the midbrain that plays an important role in the movement. The definitive diagnosis of the disease can be performed only in the histopathological examinations of the brain tissue after death (Jankovic and Kapadia 2001). Although the source of PD is still unclear, some studies show that it is caused by oxidative stress and mitochondrial dysfunction in neurons (Stefanis et al. 1997). To study PD

Published online: 25 March 2020

in vitro or to produce a PD cell model, there is a common method to obtain dopaminergic neuronal loss that contains the use of the neurotoxin, 1-methyl-4-phenylpyridinium ($MPP^+$), formed after MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) metabolism. $MPP^+$ is able to induce parkinsonism and transported to the mitochondria, where it inhibits complex I, interacts with mitochondrial respiration and this interaction increases the amount of superoxide anions (Singer et al. 1987). This formation damages energy production and leads to increased free radicals in the cell, eventually causing neuron death (Lee et al. 2011). As recent studies have described that $MPP^+$ toxicity on dopaminergic neurons could result in apoptosis through oxidative stress and inflammation (Esteves et al. 2008; Subramaniam and Chesselet 2013). The human embryonal carcinoma (EC) stem cell line (NTera2) was investigated to show similar characteristics with human embryonic stem cells (hESCs) (Pleasure and Lee 1993). Also, differentiation of NTera2 cell line by administration with *all-trans* retinoic acid was analyzed to give rise to human mature neuron-like cells and this cell culture was shown to be useful in many applications for in vitro brain disease studies (Lee and Andrews 1986; Zeller and Strauss 1995). Therefore, various studies recommended utilizing differentiated NTera2 cell line as in vitro Parkinson's disease model to understand the disease from different perspectives (Schwartz et al. 2005; Schlachetzki et al. 2013).

Boron is found in nature as boron salts and is one of the basic components used by plants and humans as microelements (Gerretsen and de Hoop 1954; Haas 2002). Boric acid (BA) occurs in nature as a salt compound of boron. Several studies were reported that boric acid has antioxidant (Herrero et al. 2005), anti-genotoxic (Turkez 2008), anti-carcinogenic (Turkez and Geyikoglu 2010), non-cytotoxic (Turkez et al. 2010) and metal chelating (Turkez et al. 2012; Üstündağ et al. 2014) properties. On the other hand, boron nitride (BN) consists of boron and nitrogen elements and it can occur in different forms (hexagonal, cubic, sheets etc.) at various temperatures and pressures but, under normal conditions, the most stable form of BN is the hexagonal form (Merlo et al. 2018). It was reported that boron nitride nanotubes did not show any toxic effects on the kidney cells and BN nanotubes were shown to have good biocompatibility properties (Chen et al. 2009). A recent in vivo study revealed that G-chitosan-coated BN nanoparticle has non-toxic effects on the marginal ear vein of male rabbits (Ciofani et al. 2012). Again, a recent investigation asserted that boron nitride nanoparticles showed no cytotoxic potential on cultured healthy human alveolar cells and suggested that these nanoparticles could be used in pharmacological and medical applications safely (Türkez et al. 2019a).

In this study, it was aimed to investigate the effects of well-characterized hexagonal boron nitride nanoparticles (hBNs) against $MPP^+$-induced neurotoxicity on neuron-like differentiated NT-2 cell culture to be able to explore novel uses area of boron nanoparticles in biomedical purposes. Firstly, NT-2 cells were transformed into neuron-like cell structure after treatment with all-trans retinoic acid and then an experimental PD model was formed with a certain dose of $MPP^+$ treatment. Subsequently, hBNs were administered at various doses to determine biocompatible concentrations on transformed NT-2 cells and cell viabilities were determined by various cytotoxicity assays. Oxidative alterations were assessed with total antioxidant status (TAS) and total oxidant status (TOS) tests. Acetylcholinesterase (AChE) enzyme activity was also analyzed. Finally, the effects of hBNs on $MPP^+$ induced apoptosis was determined by flow cytometry analysis.

## Materials and methods

### Synthesis and characterization of hBNs

For the synthesis of hBNs, 5 g boric acid ($H_3BO_3$) and 16 g sodium azide ($NaN_3$) was dissolved in 600 ml deionized water. The mixture was agitated in the magnetic stirrer for 30 min then; 4.6 ml of $N_2H_4.H_2O$ were added slowly. The sample was mixed in the magnetic stirrer for half an hour again. The mixture was placed in the autoclave in the presence of an inert gas. The autoclaved samples were incubated in the ash oven at 300 °C for 16 h. Then the mixture was washed with deionized water to purify and samples were dried under vacuum at 100 °C for 2–3 h.

Surface and size analysis of hBNs were performed by scanning electron microscopy (SEM) (Zeiss Sigma 300®). The primary characterization of hBNs was performed by X-ray diffraction (XRD) method. XRD measurement was carried out at the Eastern Anatolia Technology Center (DAYTAM, Atatürk University, Erzurum, Turkey) using the Empyrean® (PANalytical®, Germany) model XRD. Analyzes were executed in 0.1 steps between $2\theta = 10–90°$ and X-rays with a wavelength of 1.5406 Å.

### Cell culture and neuronal transformation

Human malignant pluripotent embryonic carcinoma Ntera-2/D1 (NT-2/D1) cells (ATCC® CRL-1973) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) inactivated fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin antibiotics. Cells were incubated at 37 °C, 5% $CO_2$ and 95% air atmosphere. When cells were reached %80 confluences in the flask, dead cells were removed from the culture via washing with phosphate buffer saline (PBS). Cultures were incubated with a new medium containing 5% FBS with 10 μM concentration of all-trans-retinoic acid (all-trans-

RA) (Sigma Aldrich®). The next day, the medium was replaced with a new medium containing 10% FBS without RA. This cycle was repeated for 11 days and changes in cells were photographed under an invert microscope (Andrews 1984; Andrews et al. 1990). Cell cycle phase distributions were investigated by using flow cytometry cell cycle analysis to confirm differentiations.

To constitute experimental Parkinson's disease model, 1-methyl-4-phenylpyridinium ($MPP^+$ iodide) (Sigma Aldrich®), an active metabolite of dopaminergic neurotoxin MPTP, was applied into differentiated cells at various concentrations (0.62, 0.12, 0.25, 0.50, 1 and 2 mM) and cell viability tests were performed after 24 h. The $IC_{50}$ concentration of $MPP^+$ was determined due to the results of viability tests. The differentiated cells were introduced with $MPP^+$ at $IC_{50}$ concentration to form the experimental PD model. hBNs nanoparticles were dispersed in deionized water and applied into the cultures in a wide spectrum range (0.19, 0.39, 0.78, 1.56, 3.16, 6.25, 12.50, 25, 50 and 100 mg/L). The untreated differentiated NT-2 cells were used as negative control and 1% Triton X-100 was used as a positive control. Each experimental sample was analyzed as triple replicates.

### MTT cell viability assay

After 24 h, 10% of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well of a 96-well plate and it's incubated for 3 h at 37 °C, 5% $CO_2$ and 95% air atmosphere. After 3 h, supernatant from wells was aspirated. Formazan crystals formed inside cells were dissolved with DMSO (Sigma-Aldrich®) and measurements were performed with a microplate reader at a wavelength of 570–630 nm (Türkez et al. 2019b).

### LDH release assay

Commercially available LDH kits (Thermo Fisher Scientific Massachusetts, USA.) were used according to the manufacturer's instructions to determine the level of LDH release in the cell cultures. At the end of 24 h, 50 μL of the supernatant on cells was transferred to a new 96-well plate, 50 μL of the reaction mixture was added and incubated for 30 min in the dark at room temperature. Finally, 50 μL of the stop solution was added to wells and it was measured at 490–680 nm wavelengths by a microplate reader (Türkez et al. 2019b).

### Total antioxidant status and total oxidant status analysis

Total antioxidant status (TAS) and total oxidant status (TOS) levels were calculated according to the manufacturer's instructions (Rel Assay Diagnostics®, Turkey) (Emsen et al. 2016, 2018a, b).

### Nuclear integrity analysis with Hoechst 33258 staining

After 24 h of incubation, the supernatant was removed and cells were washed with PBS. To fix the cells, the cultures were incubated for 30 min at +4 °C in the presence of 4% paraformaldehyde. After fixation, the cells were washed again with PBS and incubated with Hoechst 33258 (Sigma-Aldrich®) fluorescent dye in the dark for 5 min to stain the cell nucleus. Finally, the cell nuclei were analyzed under a fluorescent microscope (Leica® DM IL LED) (Alak et al. 2018).

### Measurement of AChE activity

AChE activities in the cultures were analyzed calorimetrically using the Acetylcholinesterase Assay Kit from Abcam® (Cambridge, MA, USA). According to the manufacturer's instructions, 50 μL of supernatant was transferred to a new plate and 50 μL of the master mix was added and stored for 30 min at room temperature protected from light. Finally, each plate well was measured at 410 nm wavelength by a microplate reader (Marinelli et al. 2017).

### Flow cytometric analysis

Cells were detached from the bottom of the cell culture plates by trypsinization at the end of the incubation for 24 h and collected by centrifugation. At the end of the centrifugation, the pellet was suspended in 500 μL of 1x binding buffer. Then, suspended cells were incubated with 5 μL of Annexin V-FITC dye and 5 μL of propidium iodide dye. After 5 min of incubation at room temperature in the dark, cells were analyzed by flow cytometry (CyFlow® Cube 6, Germany).

### Statistical analysis

The numerical data obtained from experiments were analyzed with GraphPad Prism® version 7.0 statistical program. One-way ANOVA, Tukey and Dunnett tests were used for multiple comparison evaluations. Statistically significance level was accepted as % 95 ($p < 0.05$).

## Results

### Characterization of hBNs

Surface morphology and elementary analyzes of nanoparticles were performed by SEM and XRD techniques. Using different scales, hBNs were imaged under a SEM microscope

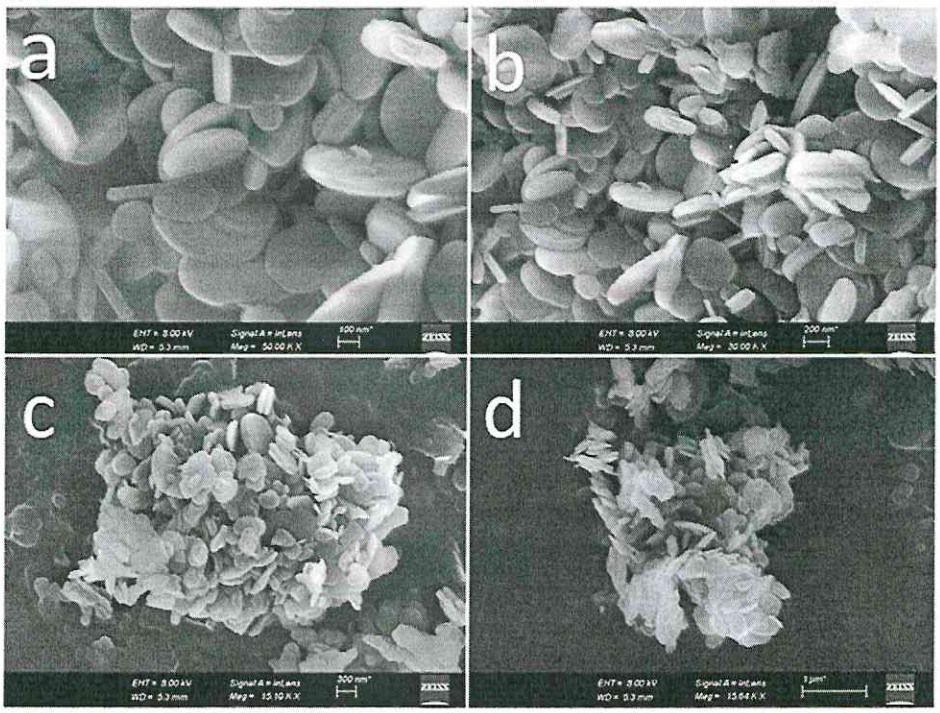


**Fig. 1** Images of hexagonal boron nitride nanoparticles (hBNs) at different scales under the scanning electron microscope (SEM); **a** 100 nm, **b** 200 nm, **c** 300 nm, **d** 1 μm

(Fig. 1). Images showed that size and dispersion of the hBNs were homogeneous and particles have a size between 100 and 400 nm.

XRD analysis of hBN nanoparticles was performed using the spray pyrolysis method previously reported by Mooney and Radding (2003). XRD analysis showed that the highest peak $2\theta = 26.76°$ corresponding to Müller index 002 plane (Fig. 2). Other peaks were observed at $2\theta =$ 41.70°, 43.91°, 55.12° and 75.97°, respectively. The planar order of these peaks according to the Müller index is 100, 101, 004 and 110. Then the size of hBNs was calculated using the Debye-Scherrer formula (Patterson 1939).

$$D = \frac{0,9\lambda}{\beta \cos\theta}$$

D represents the size of the particle, λ is X-ray beam wavelength, β is the half of the maximum radian peak value (FWHM), $\theta$ represents the Bragg angle. D value of the highest peak in 002 plane was calculated as 220 nm.

## Cell culture and neuronal transformation

NT-2 cell cultures were treated with *all-trans* RA for 11 days to differentiate into mature neuron-like cells (Esteves et al. 2015); Andrews et al. 1984; Kawasaki and Taira 2012) and cell transformations were investigated under a fluorescent microscope. After the application of RA, NT-2 cell shapes were elongated and cellular structures were developed like axon-dendrite interactions (Fig. 3). Further cell differentiation confirmations were performed by flow cytometry cell cycle analysis and according to results cell cycle tendencies were change from S phase to G1 phase (Table 1).

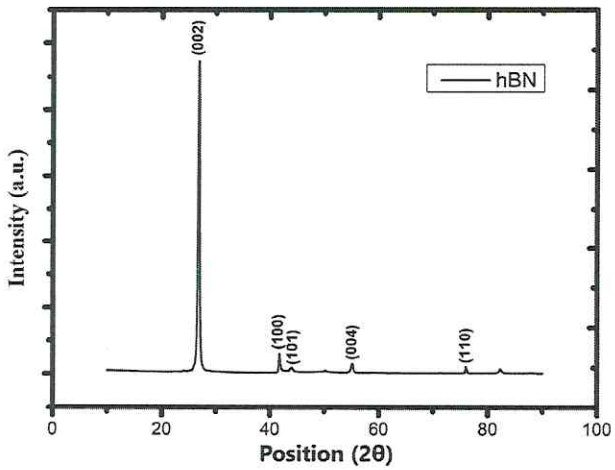


**Fig. 2** X-ray diffraction (XRD) analysis of hBNs

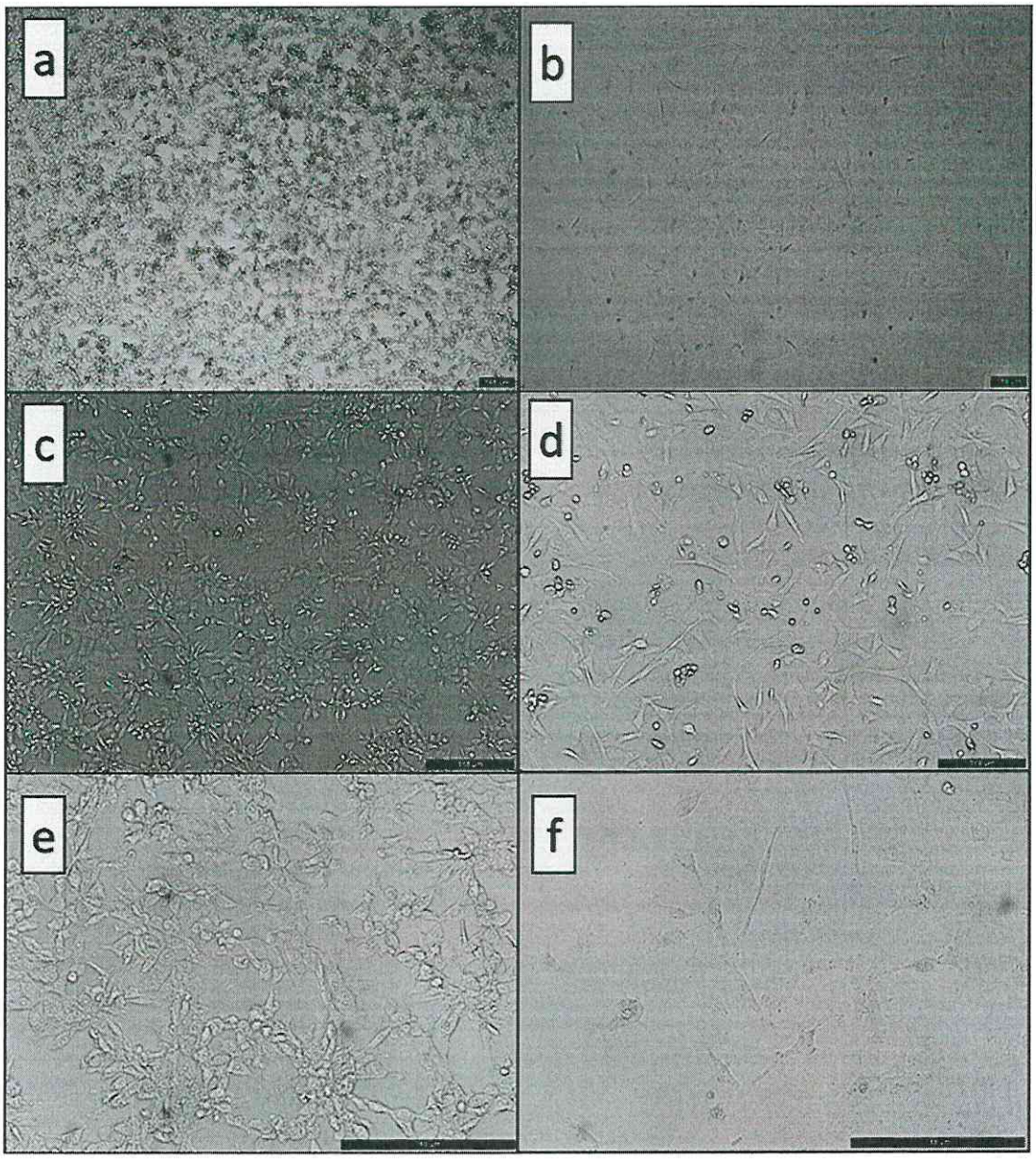


**Fig. 3** Images of undifferentiated **a** 4X, **c** 10X, **e** 20X) and differentiated **b** 4X, **d** 10X **f** 20X) culture of NT-2 cells under the microscope (after 11 days)

**Table 1** Cell cycle distribution of NT-2 cells treated with all-trans retinoic for 11 days as determined by flow cytometry analysis

| | Cell population (%) | | | |
|---|---|---|---|---|
| Group | G1 phase | G2 phase | S phase | G2/G1 |
| Control | 46,84 ± 2,15 | 15,45 ± 1,39 | 35,89 ± 2,42 | 1,82 ± 0,06 |
| RA treated | 71,35 ± 3,54* | 4,35 ± 1,32* | 20,76 ± 1,75* | 3,54 ± 0,08 |

Values are expressed as the mean ± standard deviation. Symbol (*) represents statistically significant difference compared to each phase. (GraphPad Prism® version 7.0, Two-way ANOVA, Tukey's post hoc test were used to compare the means of different values to determine significant difference ($P < 0.05$)

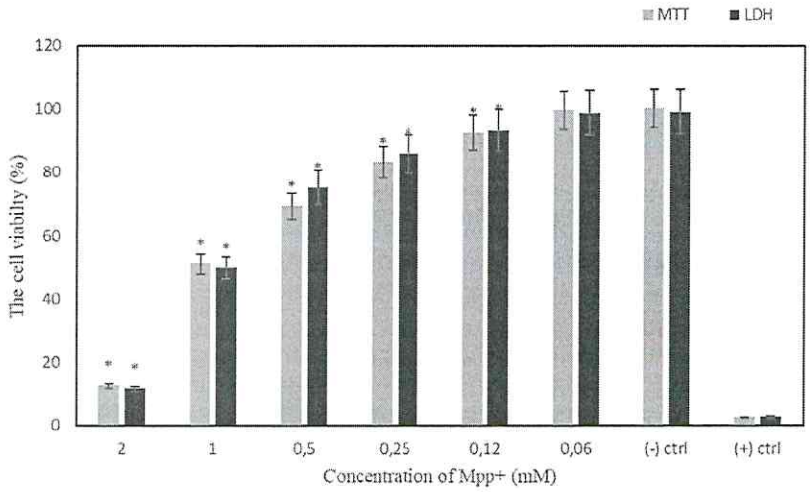


**Fig. 4** The cell viability result of differentiated NT-2 cell culture after the treated different dose manner of $MPP^+$ (24 h) (Negative control; un-treated of $MPP^+$ culture, positive control; treated %1 Triton X-100). Symbol (*) represents statistically significant decrease in cell viability at 0.12 mM concentration, (GraphPad Prism® version 7.0, Two-way ANOVA, Tukey's post hoc test were used to compare the means of different treatments to determine significant difference ($P < 0.05$)

## hBN NPs application increased cell viability in the experimental PD model

Primarily, in order to form an experimental PD model, differentiated NT-2 cells were exposed to various doses of $MPP^+$ for 24 h. At the end of 24 h, MTT and LDH release tests were applied to differentiated NT-2 cells to determine the $IC_{50}$ concentration of $MPP^+$. According to the results of MTT and LDH release tests, the $IC_{50}$ concentration of $MPP^+$ was determined as 1 mM (Fig. 4).

In this study, to explore their cytotoxic damage potential, hBNs were applied to differentiated NT-2 cultures at various concentrations (0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.50, 25, 50 and 100 mg/L). At the end of the 24-h cell viability in differentiated NT-2 cultures was calculated and presented in Fig. 5. After application with 100 mg/L of hBNs, cell viability was reduced (approximately %60) as compared to untreated cultures. However, applications with hBNs at concentrations lower than 6.25 mg/L did not lead any statistically significant ($p > 0.05$) alterations as compared to the control group.

To determine neuroprotective properties of hBNs against $MPP^+$-induced neurotoxicity in the experimental PD model, hBNs were added into the well plates at different concentrations and cell viability was calculated after 24 h. After the application of 3.12 mg/L of hBNs, the observed cell viability rates were increased (13%) as compared to $MPP^+$ applied group. Likewise, 6.25 mg/L of hBNs led to increases in cell viability by approximately 6%. In other groups, cell viability decreased in a dose-dependent manner (Fig. 6). The most effective concentration of hBNs was determined as 3.12 mg/L.

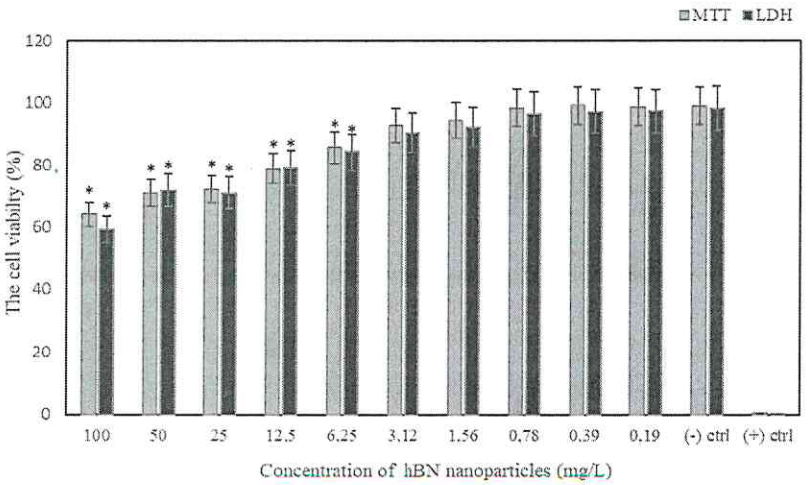


**Fig. 5** Cytotoxic effects of hBNs on differentiated NT-2 cells for 24 h. (Negative control; untreated culture, positive control; treatment with %1 Triton X-100). Symbol (*) represents statistically significant decrease in cell viability as compared to negative control group. (GraphPad Prism® version 7.0, Two-way ANOVA, Tukey's post hoc test were used to compare the means of different treatments to determine significant difference ($P < 0.05$)

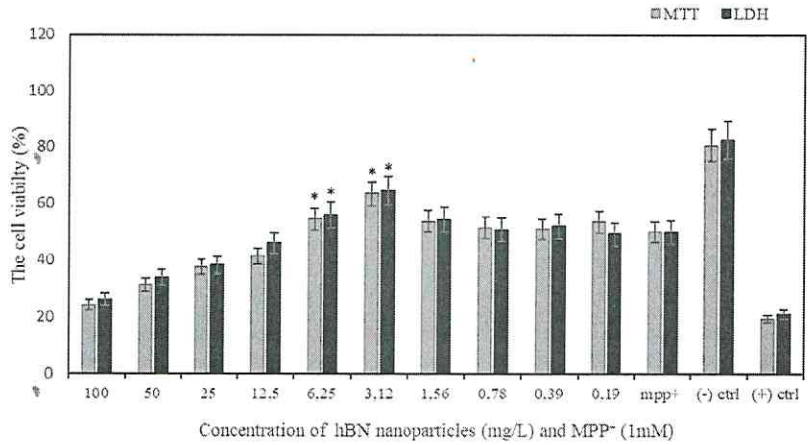


**Fig. 6** Neuroprotective potential of hBNs in experimental Parkinson's Disease model. (Negative control; untreated culture, positive control; treatment with %1 Triton X-100). Symbol (*) represents statistically significant increase in cell viability as compared to experimental Parkinson's disease model (MPP+). (GraphPad Prism® version 7.0, Two-way ANOVA, Tukey's post hoc test were used to compare the means of different treatments to determine significant difference ($P < 0.05$)

## TAS levels were increased and TOS levels were decreased against hBN applications

In this study, the effects of hBNs on oxidative alterations of neuronal cultures, TAS and TOS levels were determined. $MPP^+$ led to significant ($p < 0.05$) decreases in total antioxidant capacity and increases of the total oxidant level in differentiated NT-2 cells in comparison to untreated cultures. On the contrary, hBNs supported TAS levels without increasing the TOS levels. And, applications with hBNs led to statistically significant differences in differentiated NT-2 cells in the aspect of TAS and TOS values as compared with the positive control group (Table 2).

## hBN NPs treatment protected chromosomal integrity

To examine the effects of hBNs on chromosomal structures of cultured cells, 3.12 mg/L of hBN was applied to the in vitro PD model for 24 h. Then, cells were stained with Hoechst 33258 and examined under a light microscope and the changes in nucleus structures in the cells were photographed. It was determined that chromosomes in cells were distorted and nuclear fragments were formed in cells of the in vitro PD model. On the other hand, chromosomal integrity was found to be similar to healthy cells after applications with hBNs in contrast to the positive control. In only hBNs applied group, no significant change was observed in the chromosomal structures as compared to the control group. In the experimental PD model with the application with hBNs, it was observed that the number of chromosomal distortions significantly decreased and the number of healthy cells significantly increased when compared to the $MPP^+$ treated cultures (Fig. 7).

## AChE activity slightly altered after hBN NPs exposure

AChE enzyme activity was analyzed after application with hBNs at a concentration of 3.12 mg/L for 24 h. The results of the analysis showed that AChE activity of $MPP^+$ group was significantly decreased as compared to the untreated controls. On the other hand, there was no statistically significant difference in AChE activity between hBNs applied groups and the untreated group. AChE enzyme activity level slightly increased after application with hBNs in experimental PD model (Table 3).

## hBN NPs ameliorated apoptosis in experimental PD model

After the application of hBNs the cell death mechanisms were analyzed by flow cytometry assay. Only the $MPP^+$ application

**Table 2** Total antioxidant status (TAS) and total oxidant status (TOS) levels after treatment with hBNs and $MPP^+$ for 24 h

| Groups | TAS (mmol Trolox Equiv./l) | TOS (μmol H2O2 Equiv./l) |
|---|---|---|
| Control | $1,91 \pm 0,09^a$ | $11,88 \pm 0,59^a$ |
| $MPP^+$ | $1,13 \pm 0,05^b$ | $17,58 \pm 0,87^b$ |
| hBNs | $2,38 \pm 0,07^c$ | $9,02 \pm 0,65^c$ |
| hBN + $MPP^+$ | $1,85 \pm 0,06^a$ | $12,13 \pm 0,78^a$ |

One-way ANOVA, Dunnett tests were used for multiple comparison. Different letters in the same column presents statistical significance from each value, $p < 0.05$

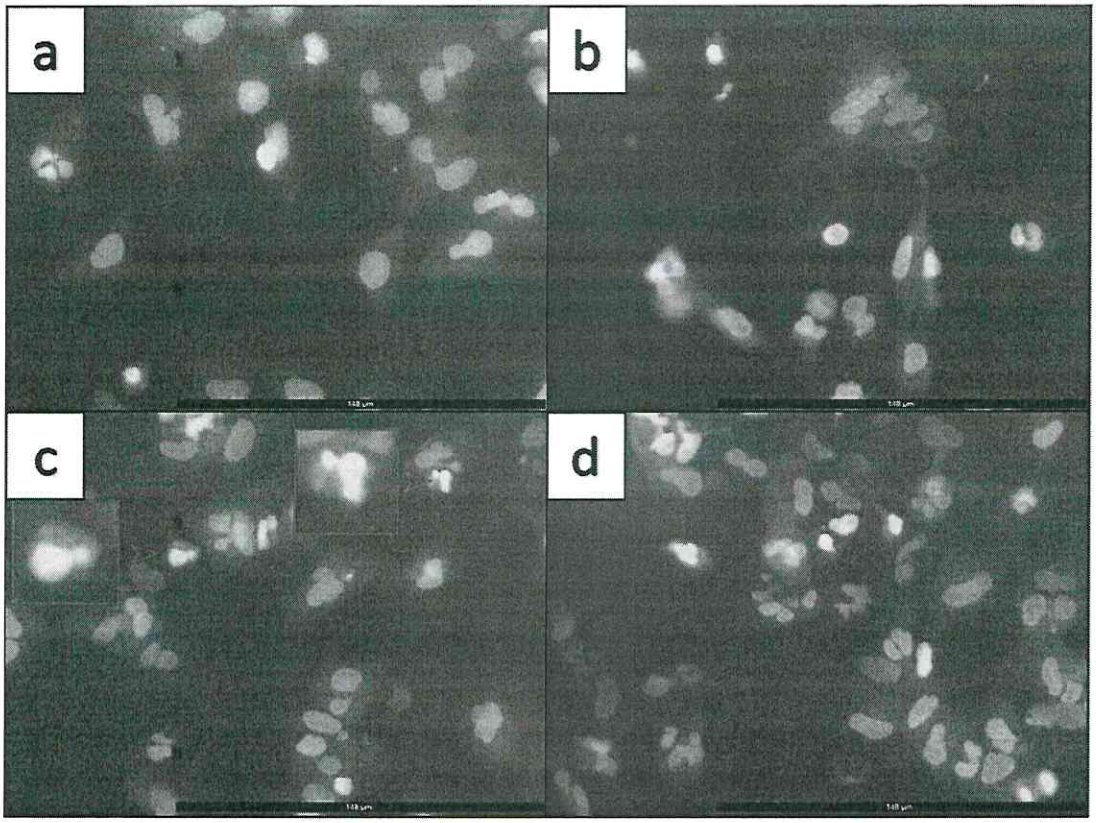


**Fig. 7** Cell nuclear staining with Hoechst 33258 (20X magnification) (24 h) **a** Control, **b** hBNs (3,12 mg/L), **c** $MPP^+$ (1 mM), **d** hBNs plus $MPP^+$

was found to induce apoptosis in the experimental PD model. However, the application of only-hBNs to differentiated NT-2 cells did not cause any change in the cell viability as compared to untreated controls. It was calculated that co-application hBNs with $MPP^+$ increased the cell viability rates from %63.49 to %93.14 as compared to only treated with $MPP^+$ (Fig. 8).

## Discussion

On XRD patterns of hBNs, there are four peaks at the Bragg angle that correspond to (002), (100), (101), (004) and (110) planes of hBNs, respectively. These peaks seen in XRD results showed that hBNs have a hexagonal structure (Korsaks 2015; Jedrzejczak-Silicka et al. 2018). Cell transformation studies have been performed for many years. RA treatment was reported to lead embryonal carcinoma cells to neuronal transformation. Again, various studies have shown that cells were transformed into neuron-like morphology and left the cell cycle by RA-induction (Jones-Villeneuve et al. 1983; Marzinke et al. 2010; Mori et al. 2013). Also, it was shown that increased Vtr protein in the Neuro2a cells after RA treatment and this increase differentiated cell morphology from multipolar to bipolar structure (Shimizu et al. 2002; Sugahara et al. 2019). In parallel to these observations, in our study, NT-2 cells were transformed into a neuron-like cell structure with all-trans RA application. $MPP^+$ which is a metabolic product of MPTP, has been discovered as a selective neurotoxin to the substantia nigra region of the brain and produces Parkinson-like toxicity (Parkinsonism) in cell cultures (Langston et al. 1984; Beal 2001; Janhom and Dharmasaroja 2015). Neurotoxin of $MPP^+$ was generally used to generate the in vitro experimental PD model (Lee et al. 2011). In this study, the experimental PD model was constituted via using the $IC_{50}$ dose of $MPP^+$ compound and resulted in apoptotic death in differentiated NT-2 cell culture.

**Table 3** The effects of hBNs and MPP+ on AChE activity for 24 h

| Groups | Acetylcholinesterase Activity (mu/ml) |
|---|---|
| Control | $431,79 \pm 4,71^a$ |
| $MPP^+$ | $355,90 \pm 6,87^b$ |
| hBN | $438,54 \pm 9,63^a$ |
| hBN + $MPP^+$ | $373,27 \pm 8,71^b$ |

One-way ANOVA, Dunnett tests were used for multiple comparison. Different letters in the same column presents statistical significance from each value, $p < 0.05$

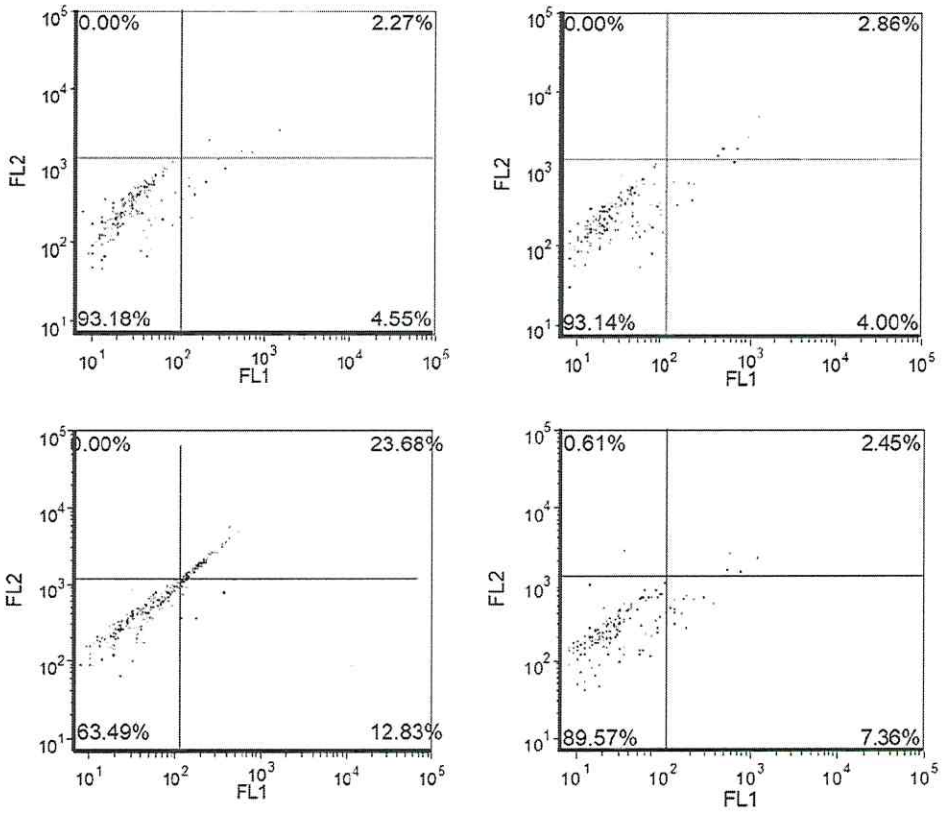


**Fig. 8** Flow cytometry results after treatment with hBNs and $MPP^+$ for 24 h. **a** Control, **b** hBNs (3,12 mg/L), **c** $MPP^+$ (1 mM), **d** hBNs plus $MPP^+$)

Recent studies showed that hBNs didn't exhibit any toxic properties on human normal skin fibroblasts and human alveolar cells at a wide spectrum of concentrations (Kıvanç et al. 2018; Türkez et al. 2019a). Our results correlated with the literature that hBNs didn't cause any toxicity at defined concentrations used in differentiated NT-2 cell culture with RA. Moreover, cell viability tests propounded that certain concentrations of hBNs were neuroprotective against $MPP^+$-induced neurotoxicity. In previous studies, it was reported that reactive oxygen species (ROS) were increased in neurodegenerative diseases, especially in PD, and consequently resulted in neuronal loss in the substantia nigra region (Kim et al. 2015; Niedzielska et al. 2016). It was found that $MPP^+$ used to form an in vitro model selectively inhibited the NADH CoQ reductase (complex I) of the mitochondrial electron transport chain (causing a dramatic reduction in ATP production) and thereby induced neuronal degeneration (Sanders and Greenamyren 2013). Furthermore, it was shown that $MPP^+$ increased $H_2O_2$-related ROS species in neurons and led to apoptosis in cell cultures (Sheehan et al. 1997; Chung et al. 2001; Shimohama et al. 2003; Janhom and Dharmasaroja 2015). According to our results, hBNs applications were significantly increased antioxidant levels, ameliorated oxidative stress and prevented apoptotic cell death caused by $MPP^+$ toxicity. Previous studies were investigated that $MPP^+$ neurotoxin reduced the AChE enzyme activity in cells in a concentration-dependent manner as compared to the control group (Zang and Misra 1993). Present results supported these data that $MPP^+$-induced neurotoxicity inhibited AChE enzyme activity in the experimental PD model. However, the application of hBNs wasn't completely successful for ameliorating inhibitory effects of $MPP^+$ on AChE activity. In this context, it can be concluded that treatment with hBNs might increase the level of antioxidants in the experimental PD model and reduce apoptosis-mediated cell death by $MPP^+$ neurotoxicity.

## Conclusions

In this study, neuroprotective properties of hBNs were demonstrated for the first time in the experimental PD model induced by $MPP^+$. Cell viability tests were determined that hBNs do not exhibit neurotoxicity potentials. Total antioxidant capacity and total oxidant status analysis showed that hBNs led to increases of TAS levels and AChE activity and decreases of TOS levels in PD model as compared to only $MPP^+$ applied group after 24 h. Finally, in the flow cytometry analysis, it was determined that the application of hBNs model significantly decreased the apoptotic cells in the experimental PD model. In the light of these findings; it can be suggested that hBNs can be used as an adjuvant or drug delivery system in the treatment of PD, since hBNs exhibit antioxidant and neuroprotective properties.

## References


Alak G, Parlak V, Aslan ME, et al (2018) Borax Supplementation Alleviates Hematotoxicity and DNA Damage in Rainbow Trout (Oncorhynchus mykiss) Exposed to Copper. Biol Trace Elem Res 1–7. https://doi.org/10.1007/s12011-018-1399-6

Andrews PW (1984) Retinoic acid induces neuronal differentiation of a cloned human embryonal carcinoma cell line in vitro. Dev Biol 103: 285–293. https://doi.org/10.1016/0012-1606(84)90316-6

Andrews PW, Damjanov I, Simon D, Banting GS, Carlin C, Dracopoli NC, Føgh J (1984) Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line Tera-2. Differentiation in vivo and in vitro. Lab Investig J Tech Methods Pathol

Andrews PW, Nudelman E, Hakomori S-I, Fenderson BA (1990) Different patterns of glycolipid antigens are expressed following differentiation of TERA-2 human embryonal carcinoma cells induced by retinoic acid, hexamethylene bisacetamide (HMBA) or bromodeoxyuridine (BUdR). Differentiation 43:131–138. https://doi.org/10.1111/j.1432-0436.1990.tb00439.x

Beal, M.F., 2001. Experimental models of Parkinson's disease., Nature Reviews Neuroscience. https://doi.org/10.1038/35072550

Chen X, Wu P, Rousseas M et al (2009) Boron nitride nanotubes are noncytotoxic and can be functionalized for interaction with proteins and cells. J Am Chem Soc. https://doi.org/10.1021/ja807334b

Chung KKK, Zhang Y, Lim KL, Tanaka Y, Huang H, Gao J, Ross CA, Dawson VL, Dawson TM (2001) Parkin ubiquitinates the α-synuclein-interacting protein, synphilin-1: implications for Lewy-body formation in Parkinson disease. Nat Med. https://doi.org/10.1038/nm1001-1144

Ciofani G, Danti S, Genchi GG, D'Alessandro D, Pellequer JL, Odorico M, Mattoli V, Giorgi M (2012) Pilot in vivo toxicological investigation of boron nitride nanotubes. Int J Nanomedicine 7:19–24

Emsen B, Aslan A, Togar B, Turkez H (2016) In vitro antitumor activities of the lichen compounds olivetoric, physodic and psoromic acid in rat neuron and glioblastoma cells. Pharm Biol 54:1748–1762. https://doi.org/10.3109/13880209.2015.1126620

Emsen B, Aslan A, Turkez H, et al (2018a) The anti-cancer efficacies of diffractaic, lobaric, and usnic acid: In vitro inhibition of glioma. J Cancer Res Ther 14:941. https://doi.org/10.4103/0973-1482.177218

Emsen B, Togar B, Turkez H, Aslan A (2018b) Effects of two lichen acids isolated from Pseudevernia furfuracea (L.) Zopf in cultured human lymphocytes. Zeitschrift fur Naturforsch - Sect C J Biosci. https://doi.org/10.1515/znc-2017-0209

Esteves M, Cristóvão AC, Saraiva T, Rocha SM, Baltazar G, Ferreira L, Bernardino L (2015) Retinoic acid-loaded polymeric nanoparticles induce neuroprotection in a mouse model for Parkinson's disease. Front Aging Neurosci. https://doi.org/10.3389/fnagi.2015.00020

Esteves ARF, Domingues AF, Ferreira IL et al (2008) Mitochondrial function in Parkinson's disease cybrids containing an nt2 neuron-like nuclear background. Mitochondrion. https://doi.org/10.1016/j.mito.2008.03.004

Gerretsen FC, de Hoop H (1954) Boron, an essential micro-element for Azotobacter chroococcum. Plant Soil 5:349–367. https://doi.org/10.1007/BF01354457

Haas ARC (2002) Boron as an essential element for healthy growth of citrus. Bot Gaz. https://doi.org/10.1086/334073

Herrero M, Ibáñez E, Cifuentes A (2005) Analysis of natural antioxidants by capillary electromigration methods. J Sep Sci 28:883–897. https://doi.org/10.1002/jssc.200400104

Janhom P, Dharmasaroja P (2015) Neuroprotective effects of alpha-mangostin on $MPP^+$-induced apoptotic cell death in neuroblastoma SH-SY5Y cells. J Toxicol. https://doi.org/10.1155/2015/919058

Jankovic J, Kapadia AS (2001) Functional decline in Parkinson disease. Arch Neurol. https://doi.org/10.1001/archneur.58.10.1611

Jedrzejczak-Silicka M, Trukawka M, Dudziak M, Piotrowska K, Mijowska E (2018) Hexagonal boron nitride functionalized with au nanoparticles—properties and potential biological applications. Nanomaterials. https://doi.org/10.3390/nano8080605

Jones-Villeneuve EM, Rudnicki MA, Harris JF, McBurney MW (1983) Retinoic acid-induced neural differentiation of embryonal carcinoma cells. Mol Cell Biol. https://doi.org/10.1128/mcb.3.12.2271

Kawasaki H, Taira K (2012) Functional analysis of microRNAs during the retinoic acid-induced neuronal differentiation of human NT2 cells. Nucleic Acids Symp Ser. https://doi.org/10.1093/nass/3.1.243

Kim GH, Kim JE, Rhie SJ, Yoon S (2015) The role of oxidative stress in neurodegenerative diseases. Exp Neurobiol. https://doi.org/10.5607/en.2015.24.4.325

Kıvanç M, Barutca B, Koparal AT, Göncü Y, Bostancı SH, Ay N (2018) Effects of hexagonal boron nitride nanoparticles on antimicrobial and antibiofilm activities, cell viability. Mater Sci Eng C. https://doi.org/10.1016/j.msec.2018.05.028

Korsaks V (2015) Hexagonal boron nitride luminescence dependent on vacuum level and surrounding gases. Mater Res Bull. https://doi.org/10.1016/j.materresbull.2015.06.032

Langston JW, Langston EB, Irwin I (1984) MPTP-induced parkinsonism in human and non-human primates–clinical and experimental aspects. Acta Neurol Scand Suppl 100:49–54

Lee VMY, Andrews PW (1986) Differentiation of NTERA-2 clonal human embryonal carcinoma cells into neurons involves the induction of all three neurofilament proteins. J Neurosci. https://doi.org/10.1523/jneurosci.06-02-00514.1986

Lee DH, Kim CS, Lee YJ (2011) Astaxanthin protects against MPTP/MPP+-induced mitochondrial dysfunction and ROS production in vivo and in vitro. Food Chem Toxicol. https://doi.org/10.1016/j.fct.2010.10.029

Marinelli L, Fornasari E, Di Stefano A, et al (2017) (R)-α-Lipoyl-Gly-L-Pro-L-Glu dimethyl ester as dual acting agent for the treatment of Alzheimer's disease. Neuropeptides 66:52–58. https://doi.org/10.1016/j.npep.2017.09.001

Marzinke MA, Henderson EM, Yang KS, See AWM, Knutson DC, Clagett-Dame M (2010) Calmin expression in embryos and the adult brain, and its regulation by all-trans retinoic acid. Dev Dyn Off Publ Am Assoc Anatomists. https://doi.org/10.1002/dvdy.22171

Merlo A, Mokkapati VRSS, Pandit S, Mijakovic I (2018) Boron nitride nanomaterials: biocompatibility and bio-applications. Biomater Sci 6:2298–2311. https://doi.org/10.1039/C8BM00516H

Mooney JB, Radding SB (2003) Spray pyrolysis processing. Annu Rev Mater Sci. https://doi.org/10.1146/annurev.ms.12.080182.000501

Mori Y, Matsui T, Omote D, Fukuda M (2013) Small GTPase Rab39A interacts with UACA and regulates the retinoic acid-induced neurite morphology of Neuro2A cells. Biochem Biophys Res Commun. https://doi.org/10.1016/j.bbrc.2013.04.051

Niedzielska E, Smaga I, Gawlik M, Moniczewski A, Stankowicz P, Pera J, Filip M (2016) Oxidative stress in neurodegenerative diseases. Mol Neurobiol. https://doi.org/10.1007/s12035-015-9337-5

Patterson AL (1939) The scherrer formula for X-ray particle size determination. Phys Rev. https://doi.org/10.1103/PhysRev.56.978

Pleasure SJ, Lee VM (1993) NTera 2 cells: a human cell line which displays characteristics expected of a human committed neuronal progenitor cell. J Neurosci Res 35:585–602. https://doi.org/10.1002/jnr.490350603

Sanders LH, Timothy Greenamyre J (2013) Oxidative damage to macromolecules in human Parkinson disease and the rotenone model. Free Radic Biol Med 62:111–120. https://doi.org/10.1016/j.freeradbiomed.2013.01.003

Schlachetzki JCM, Saliba SW, de Oliveira ACP (2013) Studying neurodegenerative diseases in culture models. Rev Bras Psiquiatr. https://doi.org/10.1590/1516-4446-2013-1159

Schwartz CM, Spivak CE, Baker SC et al (2005) NTera2: a model system to study dopaminergic differentiation of human embryonic stem cells. Stem Cells Dev. https://doi.org/10.1089/scd.2005.14.517

Sheehan JP, Palmer PE, Helm GA, Tuttle JB (1997) MPP+ induced apoptotic cell death in SH-SY5Y neuroblastoma cells: an electron microscope study. J Neurosci Res. https://doi.org/10.1002/(SICI)1097-4547(19970501)48:3<226::AID-JNR5>3.0.CO;2-H

Shimizu S, Kondo M, Miyamoto Y, Hayashi M (2002) Foxa (HNF3) upregulates vitronectin expression during retinoic acid-induced differentiation in mouse neuroblastoma Neuro2a cells. Cell Struct Funct. https://doi.org/10.1247/csf.27.181

Shimohama S, Sawada H, Kitamura Y, Taniguchi T (2003) Disease model: Parkinson's disease. Trends Mol Med. https://doi.org/10.1016/S1471-4914(03)00117-5

Singer TP, Castagnoli N, Ramsay RR, Trevor AJ (1987) Biochemical events in the development of parkinsonism induced by 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine. J Neurochem. https://doi.org/10.1111/j.1471-4159.1987.tb03384.x

Stefanis L, Burke RE, Greene LA (1997) Apoptosis in neurodegenerative disorders. Curr Opin Neurol 10:299–305. https://doi.org/10.1097/00019052-199708000-00004

Subramaniam SR, Chesselet M-F (2013) Mitochondrial dysfunction and oxidative stress in Parkinson's disease. Prog Neurobiol 106–107:17–32. https://doi.org/10.1016/j.pneurobio.2013.04.004

Sugahara M, Nakaoki Y, Yamaguchi A, Hashimoto K, Miyamoto Y (2019) Vitronectin is involved in the morphological transition of Neurites in retinoic acid-induced neurogenesis of neuroblastoma cell line Neuro2a. Neurochem Res 44:1621–1635. https://doi.org/10.1007/s11064-019-02787-4

Turkez H (2008) Effects of boric acid and borax on titanium dioxide genotoxicity. J Appl Toxicol 28:658–664. https://doi.org/10.1002/jat.1318

Türkez H, Arslan ME, Sönmez E, Açikyildiz M, Tatar A, Geyikoğlu F (2019a) Synthesis, characterization and cytotoxicity of boron nitride nanoparticles: emphasis on toxicogenomics. Cytotechnology. 71:351–361. https://doi.org/10.1007/s10616-019-00292-8

Türkez H, Arslan ME, Sönmez E, et al (2019) Synthesis, characterization and cytotoxicity of boron nitride nanoparticles: emphasis on toxicogenomics. Cytotechnology. https://doi.org/10.1007/s10616-019-00292-8

Turkez H, Geyikoglu F (2010) Boric acid: a potential chemoprotective agent against aflatoxin b1toxicity in human blood. Cytotechnology. https://doi.org/10.1007/s10616-010-9272-2

Turkez H, Geyikoglu F, Tatar A, et al (2012) The effects of some boron compounds against heavy metal toxicity in human blood. Exp Toxicol Pathol 64:93–101. https://doi.org/10.1016/j.etp.2010.06.011

Turkez H, Tatar A, Hacimuftuoglu A, Ozdemir E (2010c) Boric acid as a protector against paclitaxel genotoxicity. Acta Biochim Pol. 57:95–97 https://doi.org/10.18388/abp.2010_2378

Üstündağ A, Behm C, Föllmann W et al (2014) Protective effect of boric acid on lead- and cadmium-induced genotoxicity in V79 cells. Arch Toxicol 88:1281–1289. https://doi.org/10.1007/s00204-014-1235-5

Zang LY, Misra HP (1993) Acetylcholinesterase inhibition by 1-methyl-4-phenylpyridinium ion, a bioactivated metabolite of MPTP. Mol Cell Biochem 126:93–100. https://doi.org/10.1007/BF00925686

Zeller M, Strauss WL (1995) Retinoic acid induces cholinergic differentiation of NTera 2 human embryonal carcinoma cells. Int J Dev Neurosci. https://doi.org/10.1016/0736-5748(95)00025-C