

The Effects of Boswellia Resin Extract on Dopaminergic Cell line, SK-N-SH, against MPP⁺-Induced Neurotoxicity

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ABSTRACT

Introduction: Oxidative stress and neuroinflammation are involved in neurodegeneration procedure in Parkinson's disease. Recently, neuroprotective potential of Boswellia resin has been demonstrated. Therefore, this study examined whether administration of Boswellia resin would attenuate MPP⁺-induced neuronal death in SK-N-SH- cell line, a human dopaminergic neurons- in vitro.

Methods: Boswellia resin extract was added to culture medium (10µg/ml) before and after exposure of SK-N-SH cell line to MPP⁺ (1000µM). Cell viability and apoptosis features were assessed using MTT and Hoechst staining, respectively.

Results: Treatment with Boswellia resin 2 and 3h prior to MPP⁺ exposure and up to 60 minutes after MPP⁺ exposure significantly increased cell viability compare to untreated cells. Apoptotic features were also reduced significantly by Boswellia resin (10 µg/ml) compare to that of control untreated cells.

Discussion: Boswellia resin has neuroprotective effects on dopaminergic neurons which can be applicable in Parkinson's disease.

1. Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative diseases characterized by tremor, stiffness and slowness of motion due to dopamine (DA) depletion following dramatic loss of dopaminergic neurons in Substantia nigra. Unfortunately, this debilitating disease has no curative treatment. Neuroprotective agents might be useful via postponing the initiation of neurodegenerative processes and increasing cell resistance to oxidative stress and neuroinflammation re-

sponsible for PD (Dauer & Przedborski, 2003; Emborg, 2004; Li, et al., 2004; Toulouse & Sullivan, 2008).

Nowadays, use of herbal therapies is of interest to suppress oxidative stress and inflammatory process (Cass, 2004; Chevrier, et al., 2005). Boswellia resin has been extensively used for many centuries for various medical purposes especially for the treatment of inflammatory diseases including arthritis, asthma and Chron's disease. In recent years, neuroprotective effects and antioxidative potential of Boswellia resin has been demonstrated (Ammon, Safayhi, Mack, & Sabieraj, 1993; Moussaieff, et al., 2008; Moussaieff, et al., 2007).

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MPP⁺, an active metabolite of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), induces one of the ideal PD models via selective destruction of nigral dopaminergic neurons (Przedborski, Tieu, Perier, & Vila, 2004).

In this study we investigated the effects of *Boswellia* resin extract before and after MPP⁺ exposure of SK-N-SH neurons, a human dopaminergic cell- in vitro.

2. Methods

2.1. Materials

Petroleum ether (PE) was purchased from Merck. Dulbecco's Modified Eagle's (DMEM)/F12 1:1, Trypsin-EDTA 0.25%, Fetal Bovine Serum (FBS), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT), Hoechst 33258 and MPP⁺ iodide were purchased from Sigma.

2.2. Extraction of *Boswellia* Resin

Boswellia resin was ground and 20g of powder was extracted with 150 ml petroleum ether (three times). The extract was washed with 200 ml NaOH 5% solution (three times) and acidified with several drops of HCl (1M). The extract was then washed with saturated NaCl solution and finally dehydrated with MgSO₄ (Mousaieff, et al., 2007).

2.3. Cell Culture and Treatment

SK-N-SH cell-line was kindly provided by Dr Hadjighassem. SK-N-SH cells were cultured in DMEM/F12 supplemented with 10% heat inactivated FBS and 1% penicillin/streptomycin. Cells were maintained at 37°C in a 5% CO₂ incubator with renewal of medium every two days. After one week, cells were seeded in 96-well plates at a density of 10⁴ cells in 100µl complete medium (DMEM with 10% FBS) and were incubated overnight to attach to the wells.

In pre-treatment group, cells were treated with 10µg of *Boswellia* resin extract (BRE) 3h prior to exposure to MPP⁺ (1000 µM). In post-treatment group, BRE (10 µg) was added 30 min after MPP⁺ (1000 µM) treatment.

2.4. Cell Viability Assay

To appraise cell viability, MTT assay was performed. After treatment with BRE for 24h, MTT solution (5mg/ml) was added to each well (10 µl) and shaken for 5

min. Plates were then incubated for 4h. Then, media was replaced with equal volume of DMSO to dissolve purple formazan. Plates were shaken for 20 min and the optical density was determined at 560 nm using Dynex MRX ELISA Reader (Labequip, Canada).

2.5. Cell Death Assessment

After treatment with BRE, cells were harvested and fixed with 4% paraformaldehyde at room temperature for 30 min and then washed with phosphate buffered saline (PBS). Hoechst solution (20 µg/ml) was added to each well and incubated for 20 min. After washing with PBS, cells were analyzed with inverted fluorescent microscope (Olympus IX71, USA) at 480 nm with 200x magnification. Bright nuclei were considered as apoptotic cells. The number of cells with apoptotic morphology was counted in six randomly chosen fields per cover slip (150–200 cells).

2.6. Statistical Analysis

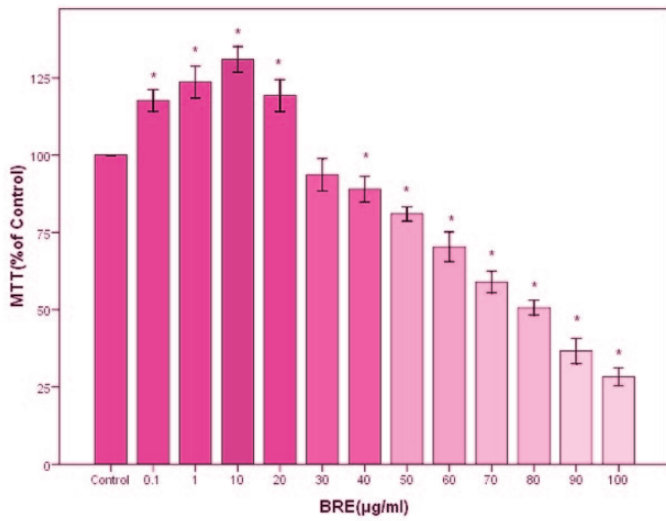
All the measurements were made in triplicate and values were represented as mean ± SEM. Statistical differences were determined with one-way analysis of variance (ANOVA) followed by Dunnett test. P values <0.05 were considered significant.

3. Results

Determination of Optimum Concentration of BRE and MPP⁺

To obtain the optimum concentration of BRE, twelve various concentrations (0.1, 1, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100µg/ml) were added to SK-N-SH cell culture medium for 24h. Results of One-Way ANOVA revealed a significant difference in percent of cell viability between concentrations less than 30µg/ml and the control untreated cells [F (12, 26)=299.7, P=.000]. Dunnett post hoc analysis showed that cell viability was increased significantly by BRE at concentrations less than 30µg/ml in comparison with control untreated cells. Concentrations more than 30µg/ml significantly decreased cell viability (P<0.05) and final concentration (100µg/ml) dramatically caused cell death. The most effective concentration of BRE that increased cell viability (10µg/ml, 131%) was selected for further experiments (fig.1).

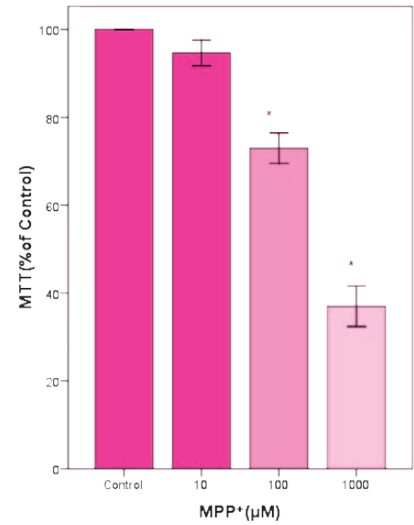
Optimum concentration of MPP⁺ was determined by testing cell viability in cells exposed to 10, 100, 1000µM of MPP⁺. Results of One-Way ANOVA revealed a significant difference in percent of cell viability



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Figure 1. Concentration-dependent effect of Boswellia resin extract on SK-N-SH cell viability in vitro.

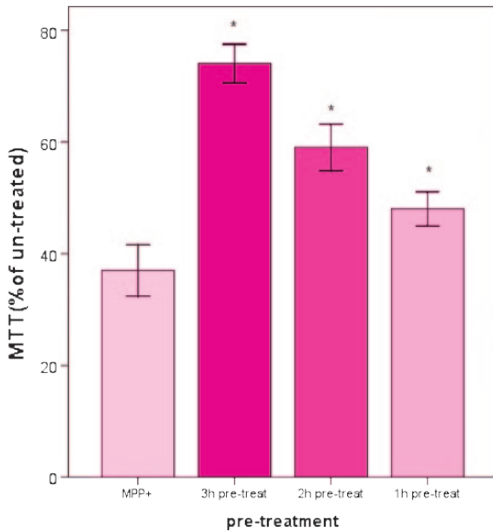
Cells were treated with various concentrations of Boswellia resin extract (0.1, 1, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/ml) for 24h and MTT assay was taken in triplicate for each concentration. Compared to control untreated cells, BRE treated cells showed higher cell viability at concentrations less than 30 µg/ml. The highest value of viability was observed at 10 µg/ml. Concentrations more than 30 µg/ml gradually decreased cell viability. Final concentration (100 µg/ml) dramatically caused cell death. The MTT values are representative of cell viability as a percent of untreated control cells. Results were mean ± SEM; *p<0.05



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Figure 2. MTT assay in SK-N-SH cells in response to MPP+.

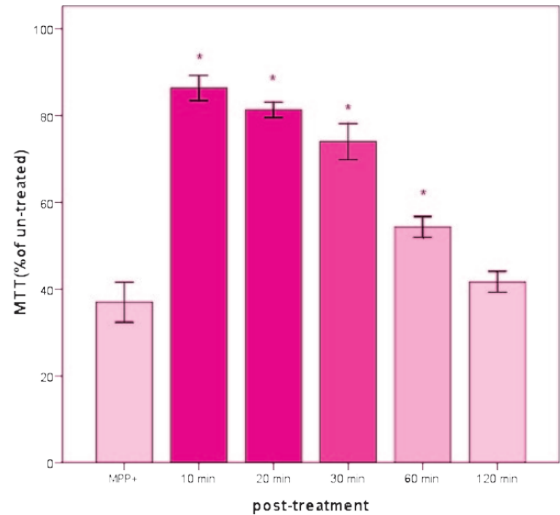
Cells were incubated in triplicate with 10, 100 and 1000 µM MPP+ for 24h. 10 µM MPP+ did not significantly decrease cell viability compared to untreated control cells whereas, 100 and 1000 µM concentrations of MPP+ critically decreased cell viability. Values are representative of mean ± SEM; *p<0.05



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Figure 3. Effect of Boswellia resin extract on cell viability in human dopaminergic SK-N-SH cells exposed to 1000 µM MPP+.

Cells were exposed to MPP+ after 1, 2, and 3hrs pre-treatment with Boswellia resin extract or without pre-treatment in triplicate. Pretreatment with BRE at 3 and 2hrs prior to MPP+ exposure increased cell viability significantly compared to cells exposed to MPP+ without pretreatment. Values are representative of mean ± SEM; *p<0.05



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Figure 4. Addition of Boswellia resin extracts at 10, 20, and 30 and 60 minute after 1000 µM MPP+ provided significant protection.

Boswellia resin extract post-treated cells at 10, 20, 30 and 60 minutes after MPP+ exposure showed increasing in cell viability from 38% to 87%, 80%, 75% and 55% of baseline. Treatment 120 min after MPP+ exposure had no effects on cell survival. Values are representative of mean ± SEM; *p<0.05

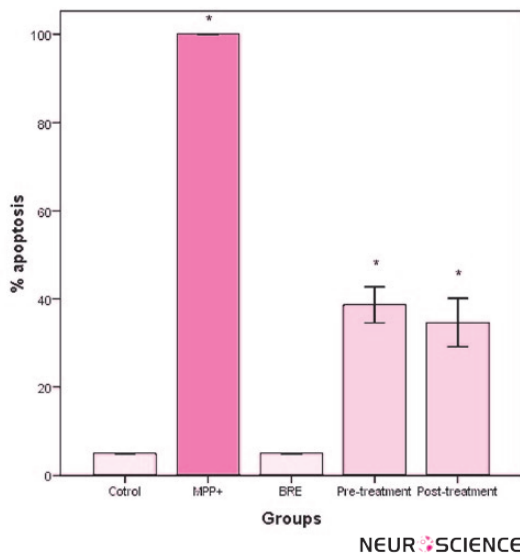


Figure 5. Apoptosis in untreated control cells and cells with *Boswellia resin extract* (10µg/ml) alone were not seen. After incubation with MPP⁺ (1000µM), condensed and shine apoptotic nuclei were observed in cells. When cells were pre-treated 3h prior or post-treated 30 minutes after MPP⁺ exposure, number of apoptotic features was significantly decreased. Values are representative of mean ± SEM; *p<0.05

ity between untreated cells and cells treated with 100 and 1000 µM MPP⁺ [F(3,8)=313.319,P=.000]. Dunnett post hock analysis showed that 100 and 1000 µM MPP⁺ decreased cell viability compare to the untreated cells. Thus, MPP⁺ at 1000µM was selected to evaluate the effects of oxidative stress on these cells (fig.2).

Effects of BRE on MPP⁺-Induced Cell Death

Exposure of SK-N-SH cells to 1000µM MPP⁺ for 24h diminished cell viability to 37% of baseline (fig.2). Cells were pre-treated with BRE 1, 2 and 3h prior to MPP⁺ exposure. Results of One-Way ANOVA revealed a significant difference in percent of cell viability [F (5, 12)=384.259, P=.000] between pre-treated and untreated cells. Dunnett post hock analysis showed that pre-treatment, two and three hours before MPP⁺ exposure, significantly increased cell viability (73% and 62% of baseline, respectively) (fig.3).

BRE had also noteworthy effects when added after MPP⁺ exposure. Cells were post-treated with BRE 10, 20, 30, 60, 120 minutes after MPP⁺ exposure. Results of One-Way ANOVA revealed a significant difference in percent of cell viability [F (7, 16)=276.035, P=.000] between cells treated with BRE up to 60 minutes after MPP⁺ exposure and untreated cells. Dunnett post hock

analysis showed that post-treatment with BRE up to 60 minutes after MPP⁺ exposure provided significant protection against MPP⁺ toxicity (fig.4).

Effects of BRE on MPP⁺-Induced Apoptosis

BRE (10µg/ml) treatment alone did not show any signs of apoptosis using Hoechst 33258 staining. Results of One-Way ANOVA revealed a significant difference in percent of apoptotic features [F (4, 10) =418.966, P=.000] between SK-N-SH-cells treated with BRE 3h before or 30 min after MPP⁺ exposure. Dunnett post hock analysis showed that MPP⁺ exposure (1000µM for 24h) significantly increase the number of nuclei with apoptotic features (e.g. highly clumped and fragmented chromatin) while control untreated cells did not show fragmented nuclear DNA (fig.5 and 6).

4. Discussion

In the present study, the possible neuroprotective effects of BRE against MPP⁺ were investigated, using SK-N-SH cell line, as dopaminergic neurons. Our results show that BRE increases cell viability and also decreases cell apoptosis before and after exposure to MPP⁺. This might indicate that BRE exerts neuroprotective effects on these cells.

Our results indicate that BRE has neurotrophic effects on human dopaminergic SK-N-SH cell-line and induces neurite outgrowth. We demonstrated that when BRE was added to culture medium alone, it improved life quality of neurons at low dose (<30µg/ml). As a result, we could suggest that BRE act as a neurotrophic agent in a certain concentration. BRE might rise neurons viability directly or activate endogenous factors like BDNF and GDNF, which can be explored by further studies.

Our results indicate that high concentration of BRE (>30µg/ml) results in cell damage and reduces cell viability. Therefore, beneficial effects of herbal agents like BRE should be defined similar to chemical agents and their limitations should be noted.

MPP⁺ has high affinity to DA receptor and induces oxidative stress (Przedborski, Tieu, Perier, & Vila, 2004) and activates molecular pathways of apoptosis (Humphrey, Cole, Pendergrass, & Kinningham, 2005; Yang, He, & Zhang, 2002) with entrance to dopaminergic neurons and inhibition of complex I of mitochondrial respiratory chain. Moreover, inflammation process occurs and inflammatory cells such as microglia cells are activated. Cell toxicity of MPP⁺ was shown before

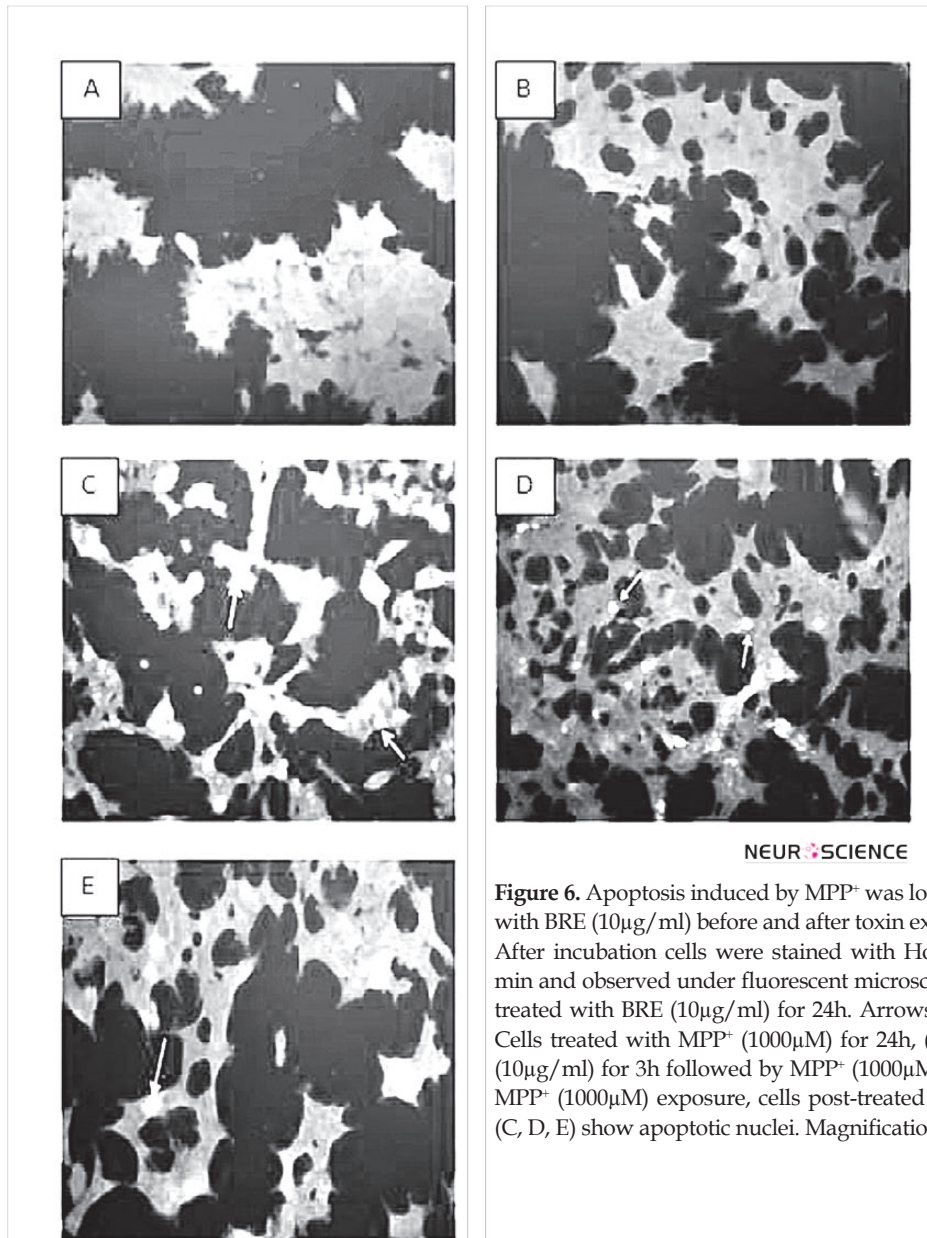


Figure 6. Apoptosis induced by MPP⁺ was lowered when cells were treated with BRE (10 µg/ml) before and after toxin exposure. After incubation cells were stained with Hoechst 33258 (20 µg/ml) for 30 min and observed under fluorescent microscope. (A) Control cells, (B) cells treated with BRE (10 µg/ml) for 24h. Arrows show neurite outgrowth. (C) Cells treated with MPP⁺ (1000 µM) for 24h, (D) cells pre-treated with BRE (10 µg/ml) for 3h followed by MPP⁺ (1000 µM) for 24h and (E) 30 min after MPP⁺ (1000 µM) exposure, cells post-treated with BRE (10 µg/ml). Arrows (C, D, E) show apoptotic nuclei. Magnification: 200X.

by Mathiasen and colleagues (Mathiasen et al., 2004). They indicated that 1mM MPP⁺ for 24h causes significant diminish in cell viability. In this study, we showed that *Boswellia* resin extract attenuates MPP⁺-induced cell death in SK-N-SH cell-line.

Our results reveal that potential effects of BRE are more than its antioxidative activity demonstrated before by Mothana and colleagues (Mothana, Hasson, Schultze, Mowitz, & Lindequist, 2011). We observed that even low concentration of BRE can rescue SK-N-SH cells against the highest concentration of MPP⁺. Thus we conclude that BRE protects SK-N-SH cells via other mechanisms. Moussaieff and colleagues in 2007 indi-

cated that incensole acetate- an active component of *Boswellia* resin- suppresses inflammation and releases inflammatory cytokines via inhibition of inflammatory genes initiator, NF-κB (Moussaieff, et al., 2007). Increasing evidence suggests that NF-κB, an inducible gene in response to a various stress stimuli, may involve in PD pathogenesis. Thus, incensole acetate was introduced as a novel neuroprotective agent (Moussaieff, et al., 2008). This finding was in agreement with our results.

Our results revealed that BRE protect and improve dopaminergic neurons cell viability even up to 1h after MPP⁺ administration. This may show therapeutic effect of BRE that can suppress neurodegenerative processes

responsible for PD pathogenesis. However, this beneficial effect is time dependent.

Previous studies have demonstrated that neurotoxins such as MPP⁺ induce biochemical, histochemical and morphological changes of apoptosis in neurons (Lev, Melamed, & Offen, 2003). It has also been shown that exposure of SK-N-SH cell-line to oxidative stress, activates caspase 3 and 9 and results in significant increase in number of apoptotic cells (Baratchi, Kanwar, & Kanwar, 2011). Our study showed that incubation of SK-N-SH cells with MPP⁺ provoked cell shrinkage and nuclear condensation. Whereas, number of apoptotic cells was decreased remarkably by BRE in pre- and post-treated groups. These results demonstrate that BRE also has anti-apoptotic ability that might be via regulation of cell death-related gene expression.

Taking together, BRE can be introduced as a neuroprotective agent that exerts its invaluable effects by increasing cell viability and decreasing vulnerability of these cells to degenerative neurotoxicity and apoptosis processes. We suggest further studies to explore the underlying mechanisms responsible for neuroprotective effects of BRE. We also recommend testing protective effects of BRE in animal models of PD.

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