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Antiapoptotic role of Agaricus blazei extract in rodent model of Parkinson's disease

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1. ABSTRACT

Rotenone is a pesticide that has been shown to induce the pathological symptoms of Parkinson's disease (PD) in both cellular and animal models. In this study, we investigated the protective effect of Agaricus blazei extract on rotenone-induced dopaminergic degeneration and apoptosis in mice model. A. blazei extract blocked the rotenone-mediated diminution of dopamine transporter (DAT) and vesicular monoamine transporter 2 (VMAT 2) expression and the downregulation of Bcl-2 and the upregulation of Bax, caspases-3, -6, -8 and caspase-9. Present data suggest that A. blazei extract plays a crucial role in regulation of proteins expression such as DAT and VMAT2 and pro-apoptotic and anti-apoptotic in Parkinsonism. In conclusion, the present study shows that A. blazei extract act as potential neuroprotective agent in the management of Parkinsonism.

2. INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease (AD) that mainly affects the movement in elderly population. PD is characterized by tremor, rigidity, akinesia and postural instability, which arises largely due to the massive loss of dopaminergic (DA-ergic) neurons projecting from the substantia nigra (SN) to the striatum (ST) (1). PD affects about 1% of the population over 60 years of age and its incidence increases to 3% of the population over 80 years (2). In 2005, the estimated number of PD cases worldwide was about 4.4. million (3) and by the year 2030, this number will be expected to get doubled to about 9 million, based on the expected growth of the population over the age of 60.

Though the cause of PD is not known, most of the knowledge about PD pathology is gathered from various *in vivo* and *in vitro* models involving 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), paraquat and rotenone (4-6). Rotenone, a naturally occurring plant flavonoid and widely used pesticide, mimicked the symptoms of PD, both *in vivo* and *in vitro* conditions (7-9).

In vitro studies indicated that rotenone can easily cross the biological membranes, due to its

lipophilic nature and could access into the cytoplasm of DA-ergic neurons easily (7). Furthermore it could enter into mitochondria and inhibit mitochondrial complex I activity. It can also induce reactive oxygen species (ROS) generation, mitochondrial membrane potential loss (MMP) and release of cytochrome c (cyt-c) from mitochondria, which in turn activate the caspase cascades and finally leads to apoptosis (10). Tyrosine hydroxylase (TH) catalyzes the conversion of L-tyrosine to DOPA, which is the initial and rate-limiting step in the biosynthesis of DA (11). The loss of ability to optimally synthesize catecholamines is an important step in the progression of PD and other neurodegenerative diseases. Indeed, early loss of TH activity followed by a decline in TH protein is considered to contribute towards DA deficiency, which is widely used as a marker of dopaminergic depletion in PD (12). Presence of pathological inclusions of α-synuclein, a major component of LBs in dopaminergic cells contributes to the intra cellular neuropathological mechanisms in PD (13). Moreover rotenone is transported into dopaminergic neurons through DAT and it can be taken into cytoplasmic vesicles by the action of the VMAT-2. The combined *in vivo* assessment of DAT and VMAT-2 may provide an index of dopaminergic nerve terminal integrity and potential vulnerability of surviving neurons (14).

Symptomatic and effective treatment of PD in modern medicine is the supplementation of the DA in the form of L-dopa (15). However, long term administration typically leads to motor complications, such as L-dopa induced dyskinesia (LIDS) (16). Current pharmacological therapies for the disease are also inadequate. Regrettably, other therapeutic strategies such as neural transplantation, deep brain stimulation and stem cell transplantation remains in the experimental stage. Unfortunately effects to find effective agents that provide protection against neurodegeneration have been unsuccessful. A number of factors have been implicated in the pathogenesis of cell death in PD which includes mitochondrial dysfunction, oxidative stress, proteosome dysfunction, Lewy bodies formation and apoptosis (4), which offers resistant to therapeutic agents. Hence drugs from plant origin with multiple mechanisms of pharmacological actions including antioxidant, anti inflammatory, anti apoptotic and mitochondrial protective properties, may be forward in delaying/protecting neuron from neurotoxicity.

There is a rich history of the use of natural products and their active compounds in the treatment of neurodegenerative diseases, including PD. The mushrooms have been generally considered as functional foods and reported to possess various pharmacological properties due to the presence of the active components such as the β-glucans, terpenes, phenolics, steroids, and nucleosides (17-18). Agaricus blazei Murrill (A. blazei), popularly known as sun mushroom, has been subject of great interest due to its nutritional value and having pharmacological properties against various diseases including cancer, diabetes, atherosclerosis, hypercholesterolaemia, and cardiac diseases. (19) It is rich in various antioxidant compounds including gallic acid, syringic acid, pyrogallol, and also polysaccharides. (20) It is also reported to contain more amounts of nucleosides and nucleotides, adenosine etc., (21) which are able to exert neuroprotective actions. (22) Recently, Soares *et al.* (23) reported that the oral administration of A. blazei extract offered neuroprotection against experimentally induced cerebral malaria and paracetamol injury by virtue of its antioxidant, mitochondrial protective, and anti-inflammatory properties. Thus considering, the increased neuroprotective effect of A. blazei during the progression of PD, we aimed to study the effect of this mushroom extract on dopaminergic protective and antiapoptotic properties against rotenone-induced mice model of PD.

3. MATERIALS AND METHODS

3.1. Chemicals

Rotenone was purchased from Sigma Chemical Company, Bangalore, India. Anti-Bcl-2, anti-Bax, Caspase-3, Caspase- 8, Caspase-9, DAT and VMAT-2 antibodies were obtained from Cell Signalling (USA) and b-actin antibodies were purchased from Santa Cruz Biotechnology, Inc, (USA). Anti rabbit HRP conjugated secondary antibody (Sigma chemical, USA). All other chemicals were of analytical grade.

3.2. Preparation of methanolic extract of A. blazei

Mushrooms were collected and then air dried in an oven at 38°C. For methyl alcohol extraction, 20 g of dried mushroom samples was weighed, ground into a fine powder, and then mixed with 200 ml of methyl alcohol at room temperature at 17 × g for 24 hours. The residue was re-extracted under the same conditions until the extraction solvents became colorless. The extract obtained was filtered on a Whatman no. 1 paper and the filtrate was collected, then methyl alcohol was removed using a rotary evaporator at 38°C to obtain the dry extract. The extract was placed in a plastic bottle and then stored at −80°C.

3.3. Animals and drug treatment

Male Albino mice (25–30 g) aged 10 weeks was procured from the Biogen Laboratory, Bangalore, India. They were kept under ambient conditions and fed with standard pellet and water ad libitum. All the experimental protocols conformed to the National Guidelines on the proper care and use of Animals in Laboratory Research (Indian National Science Academy, New Delhi, India, 2000) and were approved

by the Animal Ethics Committee (SJC/IAEC/2015–2016/01; Dated 05/10/2015).

3.4. Experimental group

Twenty-four animals were randomized and distributed into four groups (n = 6): Group I - control (0.1. ml of sunflower oil i.p. for 45 days), Group II - mice treated with rotenone (1 mg/kg/day i.p. in sunflower oil for 45 days), (24) Group III - mice treated with *A. blazei* extract (100 mg/kg b.w. p.o for 45 days), (24) and rotenone (as group II) and Group IV - mice treated with A. blazei extract alone (100 mg/kg). Then the animals were sacrificed. The striatum and substania nigra were procured and utilized for the protein expression studies of dopaminergic and apoptotic indices.

3.5. Western blotting

Tissue samples were homogenized in RIPA buffer and centrifuged at 10,000 rpm for 30 min to isolate the supernatant. Protein amount was estimated according to method of Lowry *et al.* (25) and the sample containing 50 lg protein was loaded onto the polyacrylamide gels. The gel was then transferred onto a nitrocellulose membrane (PALL Corporation, Biotrace). The membranes were incubated with the blocking buffer containing 5 % non-fat dry milk powder or BSA for 2 h to reduce non-specific binding sites and blots were probed with various antibodies: Caspase-3, Caspase-8, Caspase-9, Bax, Bcl-2, β-actin (1: 2000) and DAT and VMAT 2 (1:1000) with gentle shaking overnight at 4 C. After this, membranes were incubated with their corresponding secondary antibodies (anti-rabbit IgG conjugated to HRP) for 2 h at room temperature. The membrane was washed thrice with TBST for 30 min. Immunoreactive protein was visualized by the chemiluminescence protocol (GenScript ECL kit, USA). Densitometric analysis was performed with a computer using a gel image analysis program. The data were then corrected by background subtraction and normalized against β-actin as an internal control.

3.6. Statistical Analysis

Statistical analysis was performed by one-way analysis of variance followed by Duncan's multiple range test (DMRT) using Statistical Package for the Social Science (SPSS) software package version 15.0. All data are expressed as mean ± SD for six rats in each group. Results were considered statistically significant at $p<0.05$.

4. RESULTS

4.1. Effect of A. blazei extracts on DAT and VMAT2 Expressions

To find out the protective effect of A. blazei extract against rotenone induced neurodegeneration, the expression pattern of phenotypic markers (DAT and VMAT2) in SN and ST was analyzed by Western blotting. Rotenone treatment significantly alleviated the expression of DAT and VMAT2 in both SN and ST compared to control group (*$p < 0.05$). Meanwhile, treatment with A. blazei extract reinstated these protein expressions distinctly as compared to rotenone group (#$p < 0.05$). There were, however, no significant changes between control and A. blazei extract treated groups (Figure 1).

4.2. A. blazei extract effect on rotenone induced apoptotic gene expressions

Mice treated with chronic rotenone manifested significant induction in the expression of Bax and depletion in the expressions of Bcl-2, Caspases-3, -6, -8 and -9 in SN as compared to control animals (*$p < 0.05$). Meanwhile, these alterations were significantly attenuated by co-treatment with A. blazei extract when compared to rotenone alone-treated animals (#$p < 0.0.5$). The results also revealed that prolonged treatment of A. blazei extract to mice had no significant changes in the expression of pro- and anti-apoptotic markers as compared to the control mice (Figure 2).

5. DISCUSSION

Corona *et al.* (26) demonstrated that the i.p. administration of rotenone resulted in loss of TH-neurons in SN with significant motor defects. In the experimental PD animals, decrease in the activity of TH and the dramatic drop in the expression of TH have been suggested to be of underlying importance in the pathogenesis of PD (27). It is suggested that the decrease in nigral DA caused by rotenone coincides with the enzymatic inactivation of TH with affecting the actual level of TH protein expression and cell counts. The key aspect of our study was that gavage administration of A. blazei was able to save TH expression in cells during a period when rotenone treatment alone would have abolished it. Furthermore, S-glutathionylation of TH enzyme has been suggested to be accelerated by ROS (28). In fact, it was reported that antioxidants exert a protective effect on TH immunoreactivity (29). Oral treatment of *A. blazei* may enhance TH expression in ST and SN may be due to its antioxidative property (30).

DAT is a critical regulator of DA distribution within the brain and is also a crucial determiner of the neurotoxicity of various toxins (4). Although DAT expression is essential for normal DA neurotransmission, it also prevents the entry of toxin. Degeneration of dopaminergic neurons in PD may lead to decrease in DA storage efficiency due to the decreased DAT population on the depleted dopaminergic neurons (31). The nigrostriatal system has more and heterogeneous DAT distribution and the transporter is found on plasma membranes of

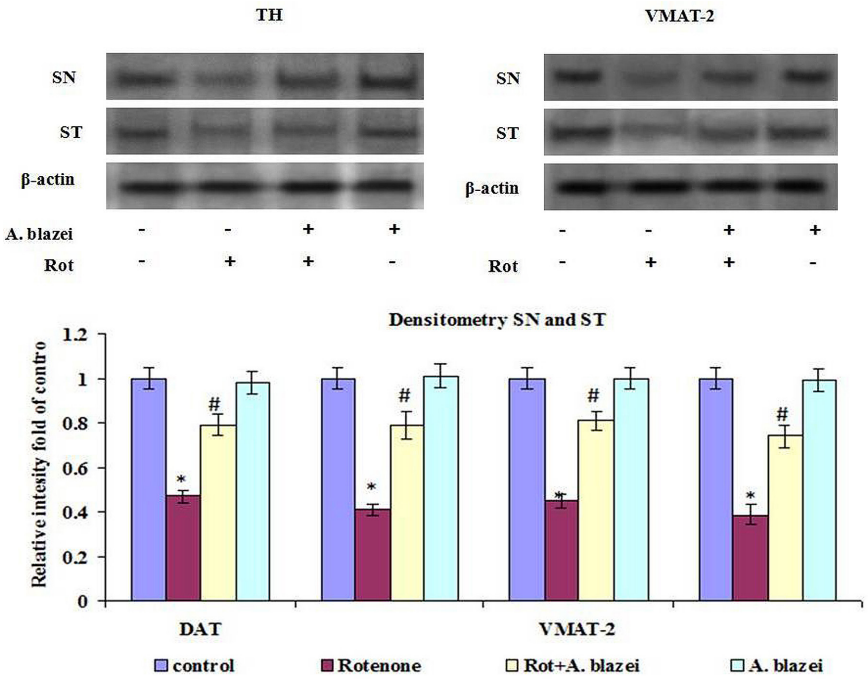

Figure 1. Effect of A. blazei on rotenone induced DAT and VMAT2 in SN and ST of experimental animals. Injection of rotenone significantly reduced DAT, and VMAT2 expressions in SN and ST. Pretreatment with A. blazei significantly increased DAT, and VMAT2 expression. Protein expressions were quantified using β-actin as an internal standard and values are expressed as arbitrary units and given as mean ± SD. P*< 0.0.5 compared to control, #p < 0.0.5 compared to rotenone.

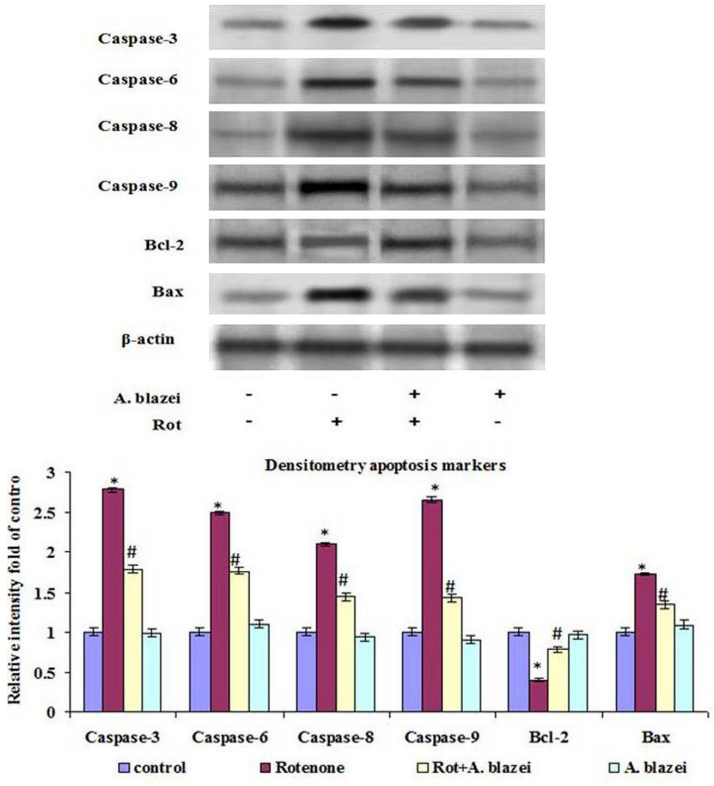

Figure 2. Effect of A. blazei on rotenone induced apoptosis in experimental animals. Administration of rotenone significantly increased the expression of pro-apoptotic proteins Bax, Caspase-3, -6, -8 and -9 and decreased the expression of anti-apoptotic protein Bcl-2. Pretreatment with A. blazei attenuated apoptosis by decreasing the expressions of pro-apoptotic markers. Protein expressions were quantified using β-actin as an internal standard and values are expressed as arbitrary units and given as mean ± SD. *p < 0.0.5 compared to control, #p < 0.0.5 compared to rotenone group.

axon terminals (32). Decline in the DAT expression is due to the loss of dopaminergic cells and fibers as a consequence of rotenone treatment in rats (24). Our western blot analysis indicated that declined expression of DAT in SN of rotenone treated mice, whereas oral administration of A. blazei extract led to increased protein expression levels of DAT in the SN as compared to rotenone alone group, which may be due to its neuroprotective effect.

Analyzing the VMAT-2 protein expression is a reliable indicator of vesicular concentration and provides a more accurate measure of nerve terminal density compared to other phenotypic markers such as DAT and TH protein expression levels (32). VMAT-2 serves as a neuroprotective factor by sequestering neurotoxin into vesicles and a critical regulator of cytoplasmic DA levels and dopaminergic function. Chen *et al.* (33) reported that the loss of VMAT-2 action in dopaminergic neurons may be a precursor to PD. Therapeutic strategies to prevent degradation of VMAT-2 or restore its function may be fruitful areas of investigation in PD research. Our western blot analysis showed the diminution of VMAT-2 expression in SN of rotenone treated mice, however, co-administration of A. blazei significantly attenuated rotenone induced neurotoxicity via enhanced VMAT-2 expression in mice.

α-synuclein is abundant in neuronal cytosolic proteins enriched at presynaptic terminals and are thought to be involved in synaptic function and plasticity (34). It is a major component of LBs and neurites, and present abundantly in LBs (35). The role of α-synuclein in normal cell function and in neurodegeneration have not been elucidated elaborately, but its potential roles in synaptic plasticity (36), neuronal differentiation, the up-regulation of DA release and mitochondrial deficits (37) have been reported. Previous findings imply that rotenone induced α-synuclein aggregation is probably mediated by oxidants generated from ROS generation (38). Because α-synuclein may be selectively and specifically nitrated, and it may link oxidative and nitrative damage to the onset and progression of neurodegenerative synucleinopathy lesions (39). Moreover, it was reported that oxidative stress can drive to α-synuclein aggregation and inclusion formation in cellular models (40). Rotenone leads to upregulation of α-synuclein expression, where as oral administration of A. blazei to PD mice partially rescued the level of α-synuclein.

During normal ageing, the rate of neuronal apoptotic cell death in dopaminergic neurons lies between 0.5. and 0.7.% per year and that the number of dopaminergic neurons is around 3,00,000-4,00,000 at the beginning of degeneration, one expects five to 10 dying neurons every day. In PD brains, the estimated rate of cell death could be around 5% per year and a maximum of 100 apoptotic neurons could be detected in the SN of PD patients (41). Apoptosis has recently been recognized as an important mode of cell death in PD (42). This has mainly been discovered by the identification of key markers of apoptotic cell death including mitochondrial Cyto-C release, alterations in Bax/Bcl-2 ratio, activation of caspases and DNA fragmentation in PD (42). Bcl-2 an antiapoptotic protein, appears to directly or indirectly preserve the integrity of the outer mitochondrial membrane, thus preventing Cyto-C release and mitochondria mediated cell damage initiation, whereas the pro-apoptotic protein, Bax, promotes Cyto-C release from mitochondria (43). Dhanalakshmi *et al.* (24) reported that rotenone treatment increased the expression of Bax protein and decreased Bcl-2 expression, resulting in imbalance between Bcl-2/Bax. This was also in concurrence with our results. Bax is translocated to the mitochondria, which results in increased colloidal osmotic pressure and mitochondrial swelling (the inner membrane cristae unfolded), rupturing of outer mitochondrial membrane and ultimately Cyto-C release (44), thereby leading to the activation of various caspases (45).

Role of caspases in the pathogenesis of PD has been established on the basis of studies in both postmortem brain tissue and animal models (46). Both extrinsic pathway or death receptor pathway and intrinsic pathway or mitochondrial pathways are known to be involved in the pathogenesis of PD. In intrinsic pathway, released Cyto-C could form apoptosomes together with apoptosis-activating factor-1 (Apaf-1) and procaspase-9, leading to the activation of caspase-9 and subsequent activation of caspase-3 (47). Apaf and Cyto-C binds to procaspase 9 to form an apoptosome by activating caspases-9, leading to subsequent proteolytic activation of the executioner caspases -3, -6 and -7, ultimately resulting in apoptosis. In the extrinsic pathway, activation of caspases 8 results in proteolytic activation of the executioner caspases -3, -6 and -7 resulting in apoptosis (48). In the present study, expressions of Caspases -3, -6, -8 and -9 significantly increased in rotenone treated animals when compared to control animals, which indicate that both the intrinsic and extrinsic pathways play important roles in apoptosis. Findings of our study demonstrate that pretreatment with A. blazei to rotenone treated mice suppresses apoptosis not only by decreasing the release of Cyto-C, but also by inhibiting the activation of caspases-9, -8, -6 and -3 and increasing Bcl-2 and decreasing Bax expressions.

5. CONCLUSION

A blazei administration protects rotenone induced dopaminergic cell loss by its anti-apoptotic action. Based on results of our investigation, we speculate that A. blazei might be a promising candidate for the prevention or treatment of PD, but further clinical studies are required.

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