Anti-oxidative and anti-apoptotic neuroprotective effects of Azadiractha indica in Parkinson-induced functional damage

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Abstract. Azadiractha indica has previously been demonstrated to act as a multi-functional medicinal plant for >2,000 years in India, and its neighboring countries. Currently, it is considered a natural resource with great value used in industrial product development and as a medicine for various types of diseases. The present study investigated the neuroprotective effects of Azadiractha indica which improved functional recovery in the 6-hydroxydopamine induced rat Parkinson's disease (PD) model. Catalase, glutathione-peroxidase, tumor necrosis factor-α, interleukin (IL)-1β, IL-6, nuclear factor (NF)-κB p65, inducible nitric oxide synthase (iNOS) and AChE activity levels were analyzed via ELISA. Western blotting was used to analyze B cell lymphoma-2 associated X protein (Bax), cytochrome c and p53 protein expression. Treatment with Azadiractha indica significantly decreased the PD-induced rotational behavior in rats. PD-induced catalase, glutathione-peroxidase, iNOS activity and iNOS protein expression were significantly suppressed by treatment with Azadiractha indica. Inflammatory factors, acetylcholinesterase activity and cyclo-oxygenase-2 protein expression levels were additionally significantly suppressed by treatment with Azadiractha indica. The protein expression levels of Bax, cytochrome c and p53 were decreased and caspase-3 and caspase-9 activities diminished, with treatment with Azadiractha indica. Therefore, Azadiractha indica was demonstrated to exhibit neuroprotective antioxidative and anti-apoptotic effects in Parkinson's disease.

Introduction

Parkinson's disease (PD) is the most frequently occurring neurodegenerative disease following Alzheimer's disease (1,2). The primary pathogenesis is the loss of dopamine (DA) neurons in the substantia nigra pars compacta and the accumulation of ubiquitinated α-synuclein in the remaining nigra DA neurons (1). At present, the primary purpose of preclinical research is to identify a novel therapeutic or therapeutic target, or develop a treatment strategy to decrease the rate of and inhibit neurodegeneration (3). However, as of yet, no current drug used in clinical practice has been able to inhibit the neurodegeneration associated with the disease.

It has previously been demonstrated that oxidative stress injury is the final pathological event in the progression of PD (4). It has therefore been hypothesized that if a particular medicine may inhibit the generation of reactive oxygen species and nitric oxide and repair the damaged mitochondrial complex, it may act as a neuroprotective reagent (5).

Acetylcholinesterase (AChE) interferes via hydrolysis of the acetylcholine neurotransmitter, to terminate nerve impulses (6). It has previously been demonstrated that AChE may promote cell apoptosis and an AChE inhibitor in the treatment of Parkinson's disease may prevent apoptosis of dopaminergic neurons in vitro and in vivo by dopaminergic neurotoxicity (6).

Azadiractha indica is termed 'Arishtha' in Sanskrit which means ‘the eliminator of pain’. It was traditionally regarded as a therapeutic product in India (7). In Ayurveda medicine theory, the bark is used in tonics, as an astringent, anthelmintic and anti-pyretic. Furthermore, Azadiractha indica is an effective lipid-lowering medicine, hypoglycemic agent, immunopotentiator, hepatoprotective and is used in anti-inflammatory and anti-fertility agents (8). The aim of the present study was to evaluate the neuroprotective effects of Azadiractha indica in the functional recovery of the 6-hydroxydopamine-induced rat Parkinson's model.

Materials and methods

Animals and experimental design. All protocols were approved by the Animal Care and Welfare Committee of the Institute of Qilu Hospital of Shandong University (Shandong, China). Male Wistar rats (300-350 g, 8-10 weeks, n=30) were purchased from Vital River Laboratories Co., Ltd. (Beijing, China) and were maintained under temperature-controlled conditions with a 12 h light/dark cycle and allowed food and water ad libitum.

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Rats were divided into five groups (n=6 animals per group): i) Sham group, ii) model group, iii) 500 mg/kg azadirachta group, iv) 1,000 mg/kg azadirachta group; and v) 2,000 mg/kg azadirachta group. In the sham group, healthy rats were injected intraperitoneally with normal saline. In the model group, PD rats were injected intraperitoneally with normal saline. In the three azadirachta treatment groups, PD rats were injected intraperitoneally with 500, 1,000, and 2,000 mg/kg azadirachta respectively, for 5 days.

6-Hydroxydopamine induced rat model of PD. A total of 10 µg 6-hydroxydopamine, purity >98% (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), at a final concentration of 2 µg/µl, was dissolved in 0.1% ascorbic acid. Wistar rats were anaesthetized with pentobarbital sodium (30 mg/kg, intraperitoneally) and a burr hole was drilled and then a needle inserted into the right substantia nigra pars compacta (anterior-posterior: -5.2, lateral; +2.2, dorsal-ventral; -7.8 relative to bregma). Following infusion, the needle was kept in place for 10 min. Then, the rotational behavior of the rats, which was induced by administration of 5 µl of 6-hydroxydopamine (2 µg/µl), was tested 14 days following lesion formation. Contralateral rotational turns were recorded over a period of 30 min.

Rotational testing. Following surgery, rats underwent rotational testing and lesion measurement based on the severity of motor behavioral disorder. Each rat was rotated 360°; the ipsilateral and to the contralateral sides of the lesion of each rat was measured.

Measurements of oxidative stress and inflammation. Blood samples were collected and centrifuged at 3,000 x g for 10 min at 4°C. Then, serum was collected and used to analyze catalase (CAT; A007-1-1), glutathione-peroxidase (GSH)-PX (A005), tumor necrosis factor (TNF)-α (H052), interleukin (IL)-1β (H002), IL-6 (H007) and nuclear factor (NF)-κB p65 (H020) levels, according to the manufacturer's protocols (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Absorbance values were measured by a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) at 450 nm.

Measurements of inducible nitric oxide synthase (iNOS) and AChE activity levels. Hippocampus tissue samples were collected and homogenized in 1:3 (w/v) ice-cold radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Nanjing, China) with a protease inhibitor mixture. The concentration of protein was determined with a BCA Protein Assay kit (Pierce, Thermo Fisher Scientific, Inc.) and homogenized in 1:3 (w/v) ice-cold radiimmunoprecipitation Western blotting. Devices LLC) at 450 nm.

Absorbance (A105-1) activity levels according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute). Absorbance values were measured by a microplate Reader (Molecular Devices LLC) at 450 nm.

Western blotting. Hippocampus tissue samples were collected and homogenized in 1:3 (w/v) ice-cold radiimmunoprecipitation assay buffer with a protease inhibitor mixture. The concentration of protein was determined with a BCA Protein Assay kit (Pierce, Thermo Fisher Scientific, Inc.). Proteins (50 µg) were resolved by 10-12% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were incubated overnight at 4°C with primary antibodies against: B cell lymphoma (Bcl)-2 associated X protein (Bax, sc-6236, 1:500), cytochrome c (sc-7159, 1:500) and β-actin (sc-7210, 1:4,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Following washing 4 times with Tris buffered saline-Tween-20, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (sc-2004, 1:5,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Immunoreactive bands were visualized using an enhanced chemiluminescence detection kit (GE Healthcare Life Sciences, Shanghai, China) and quantified using ImageJ software v3.0 (National Institutes of Health, Bethesda, CA, USA) and an Alliance LD system (Uvitec, Cambridge, UK).

Statistical analysis. All numerical data was reported as the mean ± standard deviation using SPSS 20.0 (IBM Corp., Armonk, NY, USA, n=3) and analyzed using one-way analysis of variance and a Tukey’s post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Neuroprotective effects of Azadirachta indica improve rotational behavior in 6-hydroxydopamine induced rat Parkinson model. PD rats were used to investigate the neuroprotective effects of Azadirachta indica and it was demonstrated that it improved the rotational behavior. As presented in Fig. 1, there was a significant increase in the turning values of the PD model group, when compared with the sham group. In the 1,000 or 2,000 mg/kg Azadirachta indica treated groups, turning values significantly decreased compared with the PD model group.

Neuroprotective effects of Azadirachta indica increase CAT and GSH-PX levels in 6-hydroxydopamine induced rat Parkinson model. Furthermore, a significant decrease in CAT and GSH-PX levels of the PD model group was observed compared with sham group (Fig. 2). However, treatment with 1,000 or 2,000 mg/kg Azadirachta indica significantly increased CAT and GSH-PX levels in PD rats when compared with the PD model group (Fig. 2).

Neuroprotective effects of Azadirachta indica inhibit iNOS level in 6-hydroxydopamine induced rat Parkinson model.
When compared with sham group, iNOS activity level was significantly enhanced in PD rats (Fig. 3). iNOS activity level was then significantly inhibited by treatment with 1,000 or 2,000 mg/kg Azadirachta indica compared with PD model group (Fig. 3).

Neuroprotective effects of Azadirachta indica inhibit inflammation in 6-hydroxydopamine induced rat Parkinson model. Additionally, PD significantly enhanced TNF-α, IL-1β, IL-6 and NF-κB of p65 levels in rats compared with sham group (Fig. 4). However, treatment with 1,000 or 2,000 mg/kg Azadirachta indica significantly suppressed the PD-induced TNF-α, IL-1β, IL-6 and NF-κB of p65 levels in rats compared with PD model group (Fig. 4).

Neuroprotective effects of Azadirachta indica inhibit AChE activity in 6-hydroxydopamine induced rat Parkinson model. As presented in Fig. 5, a significant increase in AChE activity was observed in the PD model group, compared with the sham group. However, Azadirachta indica at 1,000 or 2,000 mg/kg resulted in a significant decrease in AChE activity compared with PD model group (Fig. 5).

Neuroprotective effects of Azadirachta indica inhibit cyclo-oxygenase (COX)-2 protein expression in 6-hydroxydopamine induced rat Parkinson model. As presented in Fig. 6, there was a significant increase in COX-2 protein expression in the PD model group, compared with sham group. Treatment with 1,000 or 2,000 mg/kg of Azadirachta indica significantly suppressed COX-2 protein expression in 6-hydroxydopamine induced rats (Fig. 6).

Neuroprotective effects of Azadirachta indica inhibit caspase-3 and caspase-9 activity in 6-hydroxydopamine induced rat Parkinson model. Statistical analysis indicated that caspase-3 and caspase-9 activities in PD model rats significantly increased, when compared with sham group (Fig. 7). In addition, 1,000 or 2,000 mg/kg treatment with Azadirachta indica significantly reduced the caspase-3 and caspase-9 activities in PD rats compared with PD model group (Fig. 7).

Neuroprotective effects of Azadirachta indica inhibits Bax protein expression in 6-hydroxydopamine induced rat Parkinson model. PD significantly induced Bax protein expression in rats compared with the sham group (Fig. 8). Correspondingly, treatment with Azadirachta indica significantly suppressed the protein expression of Bax in PD rats compared with PD model group (Fig. 8).

Neuroprotective effects of Azadirachta indica inhibit cytochrome c in 6-hydroxydopamine induced rat Parkinson model. Statistical analysis revealed that PD significantly activated cytochrome c protein expression in PD rats compared with the sham group (Fig. 9). Notably, treatment with Azadirachta indica significantly suppressed cytochrome c in PD rats (Fig. 9).

Neuroprotective effects of Azadirachta indica inhibit p53 protein expression in 6-hydroxydopamine induced rat Parkinson model. As presented in Fig. 10, it was observed that there was a significant increase in p53 protein expression in PD rats compared with the sham group. However, Azadirachta indica significantly suppressed p53 protein expression in PD rats (Fig. 10).

Discussion

PD is additionally termed paralysis agitans, the incidence rate of PD is as high as 10% in the population >65-years-old, and China has a similar PD incidence rate, according to an epidemiological survey (9,10). PD is a chronic progressive disease of the nervous system that results from dysfunction of the extrapyramidal system and is a neurodegenerative disease, similar to Alzheimer's and lateral sclerosis of muscular atrophy (9,10). The neuroprotective effect of Azadirachta indica improved rotational behavior in the 6-hydroxydopamine induced rat Parkinson model.
Oxidative stress is the imbalance between oxidation and anti-oxidation in cells, and results from excess active oxygen and loss of antioxidants (11,12). Oxidative stress is necessary at the optimum physiological range, to stimulate proliferation or remove the aging cell components. However, a large amount of oxidative stress may damage the normal structure of tissues or the function of cells (12). The DA metabolism level is greater than the basal level, therefore DA neurons in the substantia nigra pars compacta are particularly vulnerable to damage resulting from oxidative stress (4). The present study demonstrated that Azadirachta indica significantly increased CAT and GSH-PX levels in PD rats. Omobowale et al (7) suggested that Azadirachta indica significantly attenuates oxidative stress and inflammation in dogs.

AChE has previously been demonstrated to act as a promoting factor of apoptosis. Therefore, the targeting of AChE as a site of anti-apoptosis is a novel idea in the neuroprotective therapy of PD (13). The disequilibrium of dopamine and acetylcholine in the corpus striatum is key in the pathogenesis of PD (14). The present study demonstrated that Azadirachta indica at 1,000 or 2,000 mg/kg resulted in a significant decrease in AChE activity in PD model rats. Soares et al (15) suggested that azadirachtin results in anti-inflammatory and anti-nociceptive activities in mice via inhibition of AChE.

In the brain of PD patients, the increase of inflammation and damage of the blood brain barrier may enhance the interaction between the central nervous system and the peripheral immune system. As a result, an increased number of leukocytes enter into the brain parenchyma (16). Under inflammatory conditions, peripheral immune cells enter the central nervous system and may result in nerve inflammation and neurodegeneration via paracrine and endocrine...
signaling (17). In the present study, it was demonstrated that *Azadirachta indica* significantly suppressed the PD-induced levels of TNF-α, IL-1β, IL-6, NF-κB of p65 and COX-2 in rats. In addition, Dkhil *et al* (8) reported the suppressive
inflammatory effects of the *Azadirachta indica* extract against Eimeria papillata-induced coccidiosis.

In addition, a pathological study on the brain of PD patients suggested that NO is important in the progression of PD (18). Under physiological conditions, an appropriate level of NO in cells is very important to maintain redox balance and cell proliferation (19). However, the excess accumulation of NO may result in an imbalance of redox reactions, and NO is toxic to DA neurons. A previous study indicated that NO is involved in DNA damage, poly (ADP-ribose) polymerase 1 activation and cell death induced by 1-methyl-4-phenylpyridinium (19). In the present study, iNOS activity level was significantly inhibited by 1,000 or 2,000 mg/kg *Azadirachta indica* compared with PD rats. Kim *et al* (20) suggested that *Azadirachta indica* protects against lethal endotoxemia and sepsis in mice via suppression of iNOS and inflammatory diseases.

It has previously been demonstrated that the increase of apoptosis is the primary cause of the loss of DA neurons in the substantia nigra corpus striatum in PD patients. Bcl-2, Bax, caspase-3, cytochrome c and p53 are associated with apoptosis of DA neurons in the substantia nigra in the midbrain (21). The apoptosis of nerve cells in PD patients is regulated by a series of genes associated with apoptosis, of which the Bcl-2 gene family is considered one of the most important (22). The known members of the Bcl-2 gene family include Bcl-2, Bcl-xl, Bcl-w, p53 and other cell apoptosis inhibiting genes and Bcl-xs, Bax, Bcl-2 associated agonist of cell death, Bcl-2 antagonist/killer 1, p53 and other cell apoptosis promoting genes (23). In addition, the present study revealed that treatment with *Azadirachta indica* significantly suppressed the protein expression of Bax and p53 in PD rats. Manosroi *et al* (24) indicated that *Azadirachta indica* extracts exhibit cytotoxic and melanogenesis-inhibitory activities via suppression of caspases-3, -8, and -9 and inhibition of Bax.

The release of mitochondrial cytochrome c is additionally important in cell apoptosis (25). Cytochrome c release in the cytoplasm may trigger a cascade reaction of activation, which leads to cell apoptosis (26). The release of cytochrome c results from the increased permeability of the mitochondrial outer membrane, which subsequently mediates activity of the cell apoptotic cascade (27). As the inducing factor of apoptosis, cytochrome c activates the apoptosisome with apoptosis protease activating factor, caspase-9 precursor and ATP/dATP, and then activates caspase-3, which triggers the cascade reaction of caspases and leads to cell apoptosis (25). The present study demonstrated that *Azadirachta indica* significantly suppressed cytochrome c levels in PD rats. Manikandan *et al* (28) suggested that *Azadirachta indica* may exhibit anti-oxidant, anti-angiogenic, anti-proliferative and anti-apoptotic effects via regulation of Bax, cytochrome c and caspase-3.

In conclusion, the present study suggested that neuroprotective effects of *Azadirachta indica* resulted in functional recovery in the 6-hydroxydopamine induced rat Parkinson model and the mechanism involved suppression of oxidative stress and inflammation, and inhibition of AChE activity and apoptosis. These findings suggest that *Azadirachta indica* may act as a novel therapeutic in the future for the treatment of PD.

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**Availability of data and materials**

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

**Authors' contributions**

XX designed the experiment; LW, LM and YL performed the experiments. XX and LW conducted data analysis; XX wrote the manuscript.

**Ethics approval and consent to participate**

All protocols were approved by the Animal Care and Welfare Committee of the Institute of Qilu Hospital of Shandong University (Shandong, China).

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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