Tropical Journal of Pharmaceutical Research November 2019; 18 (11): 2331-2340
ISSN: 1596-5996 (print); 1596-9827 (electronic)
© Pharmacotherapy Group, Faculty of Pharmacy, University of Benin, Benin City, 300001 Nigeria.

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v18i11.15

Original Research Article

Azadiradione exerts anti-inflammatory and anti-oxidant effects, alleviates dopaminergic neurodegeneration and reduces α-synuclein levels in MPTP-induced mouse model of Parkinson's disease

Tao Jin[1], Xuemei Cao[2], Zongwen Gao[2], Xue-Qin Yan[3]*
[1]Department of Pharmacy, Jinan Central Hospital, Jinan, Shandong 250013, [2]Department of Pharmacy, Binzhou Central Hospital, Binzhou, Shandong 251700, [3]Department of Neurology, The Second People's Hospital of Jingmen, Jingmen, Hubei 448000, China

For correspondence: **Email: 382910396@qq.com; **Tel/Fax:** 0086-0724-6812526*

Sent for review: 11 March 2019 *Revised accepted: 21 October 2019*

Abstract

Purpose: *To determine the effects of azadiradione (AZD), a tetracyclic triterpenoid, in 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)–induced experimental rodent model of Parkinson's disease (PD).*
Methods: *C57BL/6 mice were intraperitoneally injected MPTP at a dose of 20 mg/kg body weight in saline (4 times at 2-h intervals). Azadiradione (AZD) at doses of 12.5, 25 or 50 mg/kg were administered to separate groups of mice via oral gavage for 6 days prior to MPTP injection.*
Results: *Azadiradione (AZD) reduced loss of tyrosine hydroxylase (TH)-positive neurons. TH-positive counts increased to 91.44 % on treatment with 50 mg/kg AZD. Significantly ($p < 0.05$) down-regulated α-synuclein levels were seen following MPTP induction and AZD administration. Expressions of Bax, Bcl-2 and cleaved-caspase-3 were significantly downregulated ($p < 0.05$). Treatment with AZD inhibited the translocation of Cyt-C to the mitochondria, thereby preventing activation of apoptotic cascade. Oxidative stress induced by MPTP was significantly reduced by AZD via up-regulation of glutathione levels and SOD1/HO-1 expression. Azadiradione, at a dose of 50 mg/kg, significantly ($p < 0.05$) reduced ROS levels from 210.6 19.23%, and also reduced the levels of inflammatory cytokines.*
Conclusion: *These results indicate the anti-inflammatory, anti-oxidative and neuroprotective properties of AZD in mice. Thus, AZD is a potential candidate drug for the management of PD. However, further studies are required to ascertain this.*

Keywords: *Azadiradione, Alpha-synuclein dopamine, 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), Neurodegeneration, Parkinson's disease*

This is an Open Access article that uses a fund-ing model which does not charge readers or their institutions for access and distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0) and the Budapest Open Access Initiative (http://www.budapestopenaccessinitiative.org/read), which permit unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited.

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Parkinson's disease (PD) is a highly prevalent neurodegenerative disease affecting the elderly aged over 60 years, and usually, the mean age at the time of diagnosis is 70.5 years [1]. The etiology of PD is complex and yet to be unravelled completely. However, genetic, oxidative stress, environmental and immunological factors are associated risk factors

© 2019 The authors. This work is licensed under the Creative Commons Attribution 4.0 International License

[2]. The clinical manifestations of PD are postural instability, tremors, muscular rigidity and bradykinesia. Progressive dopaminergic (DA) neuronal loss in the substantia nigra pars compacta (SNpc) and striatum, with intraneuronal proteinaceous inclusions (the lewy bodies) are the hallmark features of PD [3]. Lewy bodies are proteinaceous aggregates of mutated protein (alpha-synuclein) seen in PD and in many neurodegenerative diseases [4]. Increasing evidence suggest that changes in α-synuclein degradation pathways contribute to neuronal cell death in PD. Thus, therapies that target α-synuclein degradation could be considered as potent strategies in PD treatment [5].

Overproduction of pro-inflammatory cytokines by the cells of the CNS are implicated in PD pathogenesis. Activated microglia in the SNpc and striatum are major sources of cytokines such as interleukins (ILs) (IL-1β and IL-6) and tumour necrosis factor (TNF-α), as well as reactive oxygen species (ROS) and nitric oxide (NO) [6]. Studies have revealed increased pro-inflammatory cytokines in the CSF and in striatal tissues of PD patients [7]. Thus, compounds that exert anti-inflammatory effects could be beneficial in PD management.

The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is extensively used for experimental induction of PD in rodents [8]. It causes specific loss of DA neurons in the model which is widely used for evaluation of novel neuroprotective agents in PD treatment [9]. Administration of MPTP produces neurodegeneration and neural impairments close to those observed in PD patients [8]. Monoamine oxidase B converts MPTP to active 1-methyl-4-phenylpyridinium (MPP+) which accumulates in SNpc [10]. Active MPP+ enters the DA neurons and perturbs the electron transport chain (complex I) in the mitochondria, leading to production of ROS [11]. ROS alter mitochondrial membrane permeability, subsequently causing the translocation of cytochrome C (Cyt-C) from mitochondria to cytosol. In addition, ROS induce activation of caspase-3, thereby up-regulating the apoptosis cascade [12].

The neem tree, *Azadirachta indicia* A. Juss which is native to the Indian subcontinent is known to possess a wide spectrum of medicinal uses including antimicrobial, anti-oxidant, anti-inflammatory, antiarthritic, antipyretic, anti-hyperglycemic and antitumor effects [13]. Neem is known to be one of the richest sources of secondary metabolites in nature, specifically tetranortriterpenoids (limonoids). Azadiradione (AZD) is one of the basic limonoids. The current investigation was aimed at studying the effects of AZD on experimentally-induced PD in mice.

EXPERIMENTAL

Chemicals and antibodies

1-Methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), and AZD were acquired from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies against glial fibrillary acidic protein (GFAP), HO-1, 1L-1β,1L-6, TNF-α, cytochrome-C, Bax, Bcl-2, inducible nitric oxide synthase (iNOS) and cleaved-caspase-3 were products of Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA. Alpha-synuclein was purchased from Abcam; β-actin, tyrosine hydroxylase (TH) and SOD-1 were obtained from Cell Signalling Technology (Beverly, MA, USA). Avidin–biotin complex from Vector Labs (Burlingame, CA, USA) was used for expression analysis via immunohistochemistry. All other reagents and chemicals were of analytical grade and were purchased from Sigma-Aldrich, unless otherwise specified.

Laboratory study animals

Male C57BL/6 mice (n = 80, aged 7 - 8 weeks, 25 - 30 g) procured from the Shanghai SLAC Laboratory Animal Company, Shanghai, China (SCXK 2012–0002) were used for investigation. The animals were housed in sterile cages (n = 6/cage) under 12 h/12 h day-night cycle at mean temperature of 23 ± 1 °C and relative humidity of 55 ±10 %, and were provided free access to water and feed. The mice were acclimatised for 5 days prior to initiation of the study. The animal handling was in compliance with the Animal Care and Use Guidelines of the Institution/Hospital, and in agreement with National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996) [14].

Experimental design and drug administration

The mice were randomly divided into 6 treatment groups (n = 12 / group). Separate groups of mice were administered AZD at doses of 12.5, 25 and 50 mg/kg body weight/day via oral gavage for 6 days. Freshly prepared MPTP in saline (20 mg/kg body weight) was administered intraperitoneally, 4 times at 2-h intervals [15] on the 6th day of AZD treatment. The MPTP experimental control mice received MPTP alone, while the control group received equivalent volume of saline in place of MPTP. The AZD-alone group of mice were given AZD at a dose of 50 mg/kg/day for 6 days but were not administered MPTP.

Preparation of brain tissue

On day 7 following MPTP injections, the mice were sacrificed. The animals were anaesthetized with Zoletil (50 mg/kg; Virbac Co., TX, USA) and trans-cardially perfused with PBS and paraformaldehyde (4 %) in phosphate buffer (0.1M). The brains were excised and immediately post-fixed in 4 % paraformaldehyde in 0.1 M phosphate buffer at 4 °C overnight, and immersed in 30 % sucrose solution in 50μM PBS for cryoprotection. Coronal sections (30-μm thick) were sliced using a freezing microtome (Leica Instruments GmbH, Germany) and kept at 4 °C prior to immunohistochemistry analysis.

Immunohistochemistry

The brain tissue sections were washed with PBS and incubated with 1% H_2O_2 for 15 min to exclude any endogenous peroxidase activity. Following incubation with primary anti-TH antibody overnight, the sections were treated with secondary antibody for 40 min. The brain tissue sections were further incubated with avidin-biotinylated peroxidase complex for 40 min and then were incubated for 3 min with diaminobenzidine (DAB). Thereafter, the sections were washed with PBS and mounted on gelatin-coated slides. The sections were then dried and dehydrated with ethanol and xylene. The TH-immunopositive cells were visualised under a microscope (Olympus Microscope System BX51; Olympus, Tokyo, Japan) and quantified and analysed using Image J software (Bethesda, MD, USA).

Immunoblotting

The mice brains were rapidly excised following sacrifice and SNpc tissues were dissected and kept frozen at – 80 ℃ until used for analysis. The SNpc tissues were rinsed well with ice-cold PBS and homogenized in lysis buffer containing 50 mM Tris buffer, pH 8, NaCl (0.02 g/ml), 1% Triton-X, aprotinin (4 U/ml), 2 mM leupeptin, 100 mM phenylmethanesulfonylfluoride and 0.2% SDS. The cell lysates were centrifuged for 20 min at 13,000 g at 4 ℃. The total protein content in the lysates was determined using Bradford's method.

Preparation of tissues for assay of Bax and Bcl-2 expressions

Mitochondrial and cytosolic fractions from the SNpc tissues (n=6) were obtained using a mitochondria/cytosol fractionation kit (Biovision Inc., Milpitas, CA, USA). The nuclear fractions (n=6) from the SNpc tissues were obtained using the ProteoJET™ Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas, Vilnius, Lithuania), according to the manufacturer's instructions.

Equal quantities of protein samples (40 μg) from each experimental group were electrophoretically separated using SDS-PAGE (10 %), and the separated bands were blotted onto a nitrocellulose membrane (Invitrogen). The membranes were blocked with 5 % FBS albumin in Tris-buffered saline containing (0.01%) Tween-20 (TBST) at room temperature for 2 h. The membranes were then incubated with specific primary antibodies overnight at 4 °C. After washing with TBST, the blots were incubated with secondary antibody conjugated with HRP (Santacruz Biotechnology) for 2 h at room temperature. The positive bands were visualized and scanned using Image Master II scanner (GE Healthcare, Milwaukee, WI, USA). The densities of the immunoreactive bands were further analysed using ImageQuant TL software (GE Healthcare, Milwaukee, WI, USA). The expressions of the sample proteins were normalized to that of β-actin which served as internal control.

Assessment of oxidative stress

Brain tissue (n = 6) were homogenized using ice-cold PBS (1:10; w/v). The homogenate was then centrifuged (3000 rpm; 15 min, 4 °C) and the supernatant obtained was used for assay of ROS, MDA content and antioxidant levels. Total protein content of the supernatant was determined using Bradford protein assay kit (BioRad, Hercules, CA, USA).

Determination of ROS (reactive oxygen species)

Brain tissue ROS levels were estimated using *in vitro* ROS/RNS assay kit (OxiSelect™) (Cell Bio Labs Inc). A fluorogenic probe dichlorodihydro-fluorescin DiOxyQ (DCFH-DiOxyQ) specific to ROS/RNS was employed in the assay. The probe was stabilised first to highly reactive DCFH. This active form of the probe reacted readily with ROS and RNS and became oxidized to the highly fluorescent DCF, the intensity of which was measured at 480 nm (excitation) and 530 nm (emission) using Synergy™ 2 Multi-function Microplate Reader.

Malondialdehyde (MDA) assay

The concentrations of MDA in the brain tissue samples of the different experimental groups

were determined using MDA assay kits from Sigma-Aldrich. The MDA content in the brain tissues was expressed as nmol/mg protein.

Determination of ratio of total glutathione to reduced glutathione (GSH: GSSG ratio)

Total glutathione and GSSG content were determined in the brain tissues as a measure of the antioxidant status using Assay Kits from Beyotime Institute of Biotechnology (Shanghai, China). Absorbance was read at 412 nm using a microplate reader. Reduced glutathione (GSH) content was determined by calculating the difference between total glutathione and GSSG. Total glutathione content was expressed as nmol/mg protein, and the GSH/GSSG ratio was calculated.

Statistical analysis

The data obtained are presented as mean ± SD (n = 6). Statistical analyses were performed using SPSS software (version 22.0, SPSS Inc., Chicago, IL). Multiple group comparisons were done with one-way analysis of variance (ANOVA), followed by *post-hoc* analysis using Duncan's Multiple Range Test (DMRT). Values of $p < 0.05$ were considered statistically significant.

RESULTS

Azadiradione prevented dopaminergic neuron cell loss following MPTP-administration

Immunohistochemical analysis was performed to assess the effects of AZD on dopaminergic neuronal loss following MPTP administration, and TH-immunohistochemistry was conducted in the ST and SNpc tissues of MPTP-treated mice. The MPTP-treated mice exhibited markedly reduced number of TH immuno-positive neurons (Figure 1 A). In MPTP injected mice, the TH immuno-positive neuronal cell counts were 37.21 %, relative to the control. However, administration of AZD significantly ($p < 0.05$) prevented MPTP-induced neuronal loss. The TH-positive counts increased to 91.44 % on treatment with AZD at a dose of 50 mg/kg. Furthermore, AZD, when administered alone did not cause any neuronal loss in mice. The MPTP treatment caused decreases in the expressions of TH, similar to IHC results (Figures 1 B and 1 C). However, AZD treatment at all doses markedly enhanced TH expression, and the expression level increased to 99 % with AZD dose of 50 mg/kg, relative to 60 % in MPTP control.

Azadiradione reduced glial activation

Astroglia activation was assessed by determination of the expression of glial fibrillary acidic protein (GFAP). The results of immunoblotting revealed significantly raised ($p < 0.05$) GFAP levels following MPTP treatment (Figures 1 B and 1 D). In contrast, AZD at all the 3 administered doses resulted in significant ($p < 0.05$) reductions in GFAP protein expression, reflecting decrease in glial cell activation.

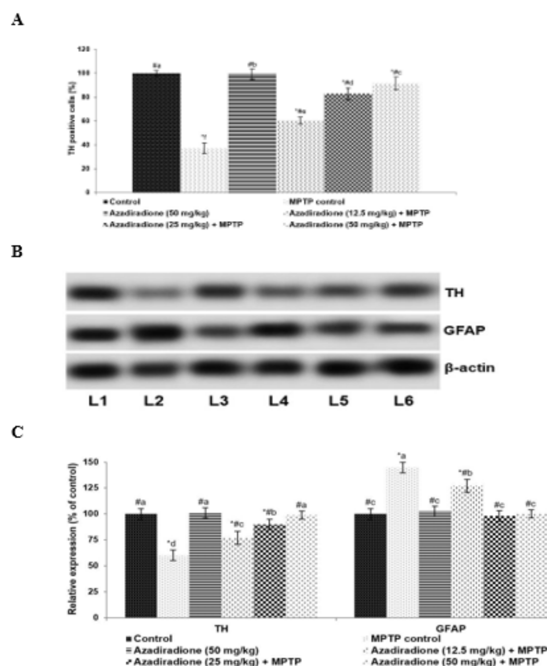

Figure 1: Azadiradione reversed MPTP-induced dopaminergic neuronal loss and reduced microglial activation. (A) TH positive cells. (B) Representative immunoblot of TH and GFAP expressions. (C) Relative expressions of proteins from different experimental groups with control expressions set at 100 %. Data are presented as mean ± SD, (n = 6); $p < 0.05$ as determined with one-way ANOVA and DMRT analysis; *$p < 0.05$ vs control; #$p < 0.05$ vs MPTP control; a - d indicate significant differences in mean values at $p < 0.05$; L1 = Control; L2 = MPTP control; L3 = AZD (50 mg/kg); L3 = AZD (12.5 mg/kg) + MPTP; L4 = AZD (25 mg/kg) + MPTP; L5 = AZD (50 mg/kg) + MPTP

Azadiradione modulated the apoptotic pathway

The MPTP administration caused significant ($p < 0.05$) up-regulation in cleaved caspase-3 levels. Interestingly, AZD suppressed caspase-3 expressions in the order 50 mg > 25 mg> 12.5 mg (Figures 2 A - C). The mitochondrial Bax expressions were markedly increased ($p < 0.05$)

along with decreased Bcl-2 levels on MPTP administration, when compared with control group (Figures 2 A -2 C). The expressions were reduced to 102 % by 50 mg/kg AZD, as against 167 % in MPTP control. Azadiradione treatment markedly ($p < 0.05$) reduced Bax and improved Bcl-2 levels, thereby restoring the Bcl-2/Bax ratio. The ratio increased to 0.72 as against 0.034 in MPTP control group. Furthermore, MPTP-induced toxicity significantly increased ($p < 0.05$) cytosolic Cyt-C level to 173 %, relative to normal control, which reflects Cyt-C translocation. However, AZD significantly ($p < 0.05$) repressed translocation of Cyt-C from mitochondria to cytosol (Figures 2 A - C). These observations indicate that AZD effectively inhibited apoptotic cascade, thereby exerting protective effects.

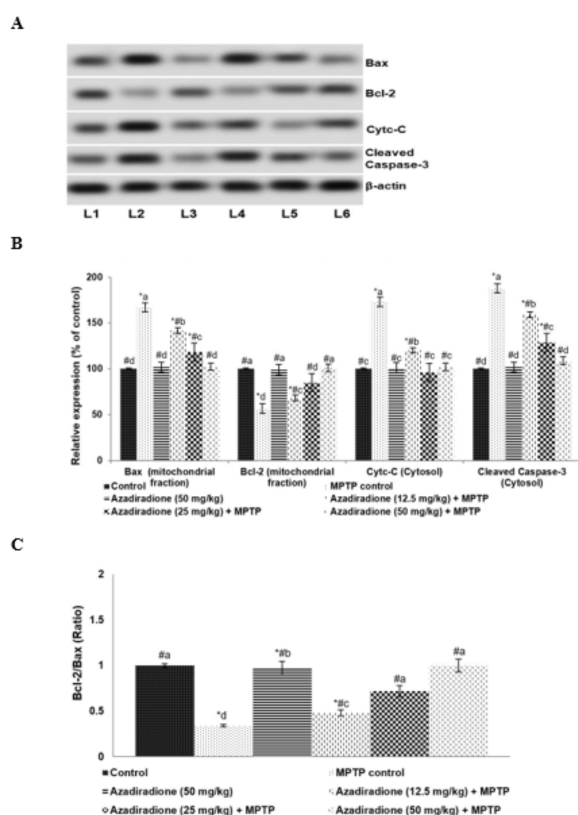

Figure 2: Effect of AZD on apoptotic pathway proteins. (A) Representative immunoblot. (B) Protein expressions from different treatment groups, relative to control expressions set at 100 %. (C) Bcl2/Bax levels. Values are presented as mean ± SD, (n = 6); $p < 0.05$, as determined with one-way ANOVA and *post-hoc* DMRT analysis. *$p < 0.05$ vs control; #$p < 0.05$ vs MPTP control; a - d indicate significant differences in mean values at $p < 0.05$; L1 = Control; L2 = MPTP control; L3 = AZD (50 mg/kg); L3 = AZD (12.5 mg/kg) + MPTP; L4 = AZD (25 mg/kg) + MPTP; L5 = AZD (50 mg/kg) + MPTP

Azadiradione reduced oxidative stress

The ROS levels are tightly regulated by antioxidant defences of the cell. Over-production of ROS and oxidative stress are associated with PD. In the present study, levels of ROS and MDA were determined in the SNpc tissues as indicators of oxidative stress. Significant ($p < 0.05$) elevations in ROS and MDA were seen on MPTP administration (Figures 3 A and B). Azadiradione treatment prior to MPTP, and after MPTP administration significantly ($p < 0.05$) reduced ROS and MDA levels (Figure 3). The ROS levels reduced to 145.08, 65.01, and 19.23 % from 210.6% on treatment with 12.5, 25 and 50 mg/kg AZD, respectively. Furthermore, AZD administration markedly raised levels of glutathione in the order 50 mg > 25 mg > 12.5 mg. Glutathione concentration remarkably increased to 55.14 nM/mg protein on treatment with AZD at a dose of 50 mg/kg, from 20.50 nM/mg protein in MPTP control (Figure 3 C). Azadiradione at all the 3 tested doses improved the altered GSH:GSSG ratio (Figure 3 D).

Expressions of enzymes of antioxidant defence system such as NAD(P)H quinone oxidoreductase-1 (NQO1), superoxide dismutase (SOD1) and heme oxygenase 1 (HO-1) were assayed. Usually, HO-1 expression is up-regulated under stress. The expressions of SOD-1, HO-1 and NQO1 were significantly ($p < 0.05$) decreased following MPTP administration (Figures 4 A and B). However, AZD treatment raised the levels of the enzymes significantly ($p < 0.05$), relative to MPTP control group. The 50 mg/kg AZD-administered group expressed HO-1 at levels almost near normal control level. Similarly, NQO1 levels were improved in a dose-dependent manner on AZD treatment, relative to MPTP control. Treatment with AZD at all 3 tested doses markedly improved expression of antioxidant defence enzymes.

Azadiradione reduced α-synuclein levels

α-Synuclein expressions in the striatum of the mice were assessed with western blotting analysis, and the results are presented in Figures 5 A and B. Accumulation of α-synuclein is a hallmark feature of PD. The MPTP administration resulted in significantly ($p < 0.05$) increased levels of α-synuclein, relative to normal control. Interestingly, AZD treatment substantially decreased the α-synuclein levels in a dose-dependent manner, with the highest dose of 50 mg/kg exerting maximal effects. The α-synuclein levels decreased from 198 % in MPTP control to 135 % with AZD treatment at a dose of 50 mg/kg.

Azadiradione down-regulated proinflammatory cytokines following MPTP-administration

Immunoblotting analysis of pro-inflammatory cytokines i.e. iNOS, IL-1β, IL-6 and TNF-α in the SNpc tissues showed that MPTP administration resulted in significant increases ($p < 0.05$) in their protein expression levels, when compared with normal control animals. Azadiradione administration prior to MPTP injection significantly ($p < 0.05$) prevented increases in IL-1β, IL-6, iNOS, and TNF-α levels, relative to MPTP control which did not receive AZD (Figure 6). At a dose of 50 mg/kg, AZD effectively reduced the levels of iNOS, IL-1β, IL-6 and TNF-α to almost near control levels.

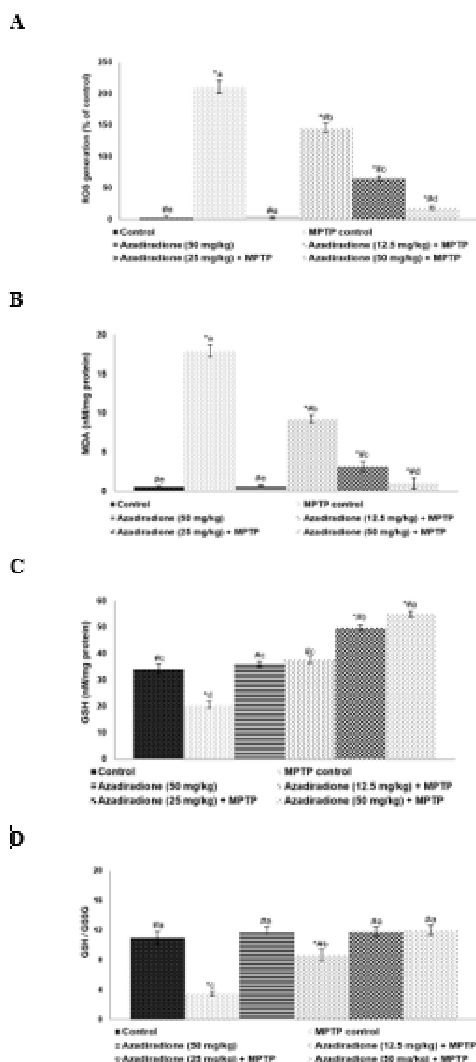

Figure 3: Azadiradione reduced MPTP-induced ROS generation and improved antioxidant levels. (A) ROS generation. (B) MDA levels. (C) GSH levels. (D) GSH:GSSG ratio. Data are presented as mean ± SD, (n = 6); $p < 0.05$ as determined by one-way ANOVA and *post-hoc* DMRT analysis. *$p < 0.05$ vs control; # $p < 0.05$ vs MPTP control; a - d indicate significant differences in mean values at $p < 0.05$

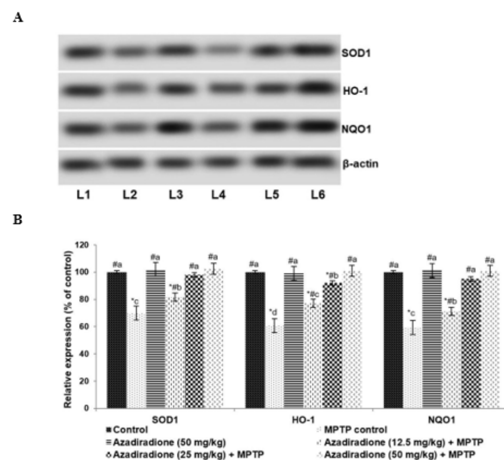

Figure 4: Azadiradione improved antioxidant levels. (A) Representative immunoblot. (B) Protein expressions in different treatment groups, relative to control expressions set at 100 %. The results are presented as mean ± SD, (n = 6); $p < 0.05$ as determined by one-way ANOVA and DMRT analysis. * $p < 0.05$ vs control; # $p < 0.05$ vs MPTP control; a - d indicate significant differences in mean values at $p < 0.05$; L1 = Control; L2 = MPTP control; L3 = AZD (50 mg/kg); L3 = AZD (12.5 mg/kg) + MPTP; L4 = AZD (25 mg/kg) + MPTP; L5 = AZD (50 mg/kg) + MPTP

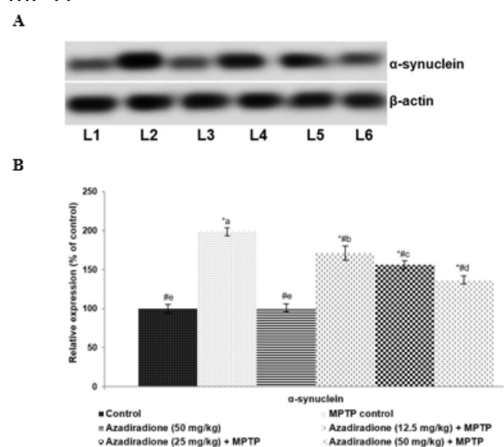

Figure 5: Azadiradione reduced α-synuclein levels. (A) Representative immunoblot. (B) α-synuclein expressions in different experimental groups, relative to control. Control expressions were set at 100 %. Values are presented as mean ± SD, (n = 6); $p < 0.05$ as determined by one-way ANOVA and by DMRT *post-hoc* analysis. * $p < 0.05$ vs control; # $p < 0.05$ vs MPTP control; a - d indicate significant differences in mean values at $p < 0.05$; L1 = Control; L2 = MPTP control; L3 = AZD (50 mg/kg); L3 = AZD (12.5 mg/kg) + MPTP; L4 = AZD (25 mg/kg) + MPTP; L5 = AZD (50 mg/kg) + MPTP.

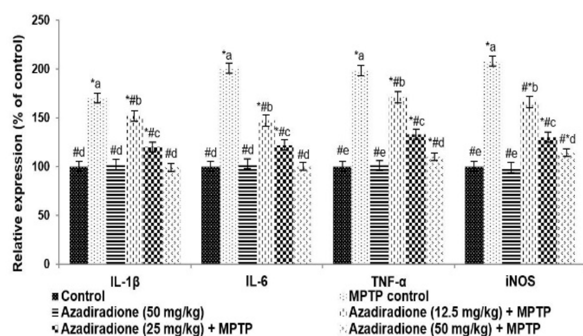

Figure 6: Azadiradione regulated the inflammatory mediators. Data are presented as mean ± SD, (n = 6); $p < 0.05$ as determined with one-way ANOVA and by DMRT analysis. * $p < 0.05$ vs control; #$p < 0.05$ vs MPTP control; a - d denote significant differences in mean values at $p < 0.05$

DISCUSSION

Parkinson's disease (PD), a neurodegenerative disorder affecting the motor system of the elderly, is characterized by progressive loss of nigrostriatal dopaminergic neurons, postural instability, muscular rigidity, bradykinesia and tremors in the limbs [16]. It is known that MPTP is a neurotoxin which is widely employed for induction of PD in experimental animal models [9]. Parkinson's disease condition is associated with accumulation of Lewy bodies, which are cytoplasmic proteinaceous inclusions composed of presynaptic protein α-synuclein [10]. It has been demonstrated that excess accumulation of α-synuclein causes dopaminergic neuronal death [17]. In this study, elevated levels of α-synuclein were observed following MPTP- administration. However, AZD administration significantly reduced α-synuclein expressions in a dose-dependent manner. It is not clear whether AZD brought about this reduction by promoting autophagy of α-synuclein aggregates or if it prevented their aggregation. However, AZD decreased α-synuclein expressions following MPTP administration.

Previous studies have reported that administration of MPTP to mice resulted in dopaminergic neuronal degeneration, as well as decline in dopamine and TH levels in the SNpc [10]. The active form of MPTP i.e. MPP+ is taken up by dopaminergic neurons. Inside the neurons, it enters the mitochondria and obstructs mitochondrial respiration complex-I [18], leading to generation of high levels of ROS [19]. Subsequently, the apoptotic pathway is triggered on via activation of caspase-3 [20].

Dopaminergic neurons are abundant in the SNpc region. The neurotransmitter dopamine is responsible for transmission of signals between SNpc and many other regions of the brain. The association between the SNpc and the striatum is vital for undisturbed, purposeful movement. Depletion of dopamine results in irregular nerve-firing in the brain, leading to impaired muscular coordination and movements. In the present study, dopaminergic neuronal loss was determined by evaluation of TH-positive cells in SNpc. Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the synthesis of dopamine. It catalyses the conversion of L-dopa to dopamine [21]. Tyrosine hydroxylase (TH)-immunoreactivity reflects the functional status of dopaminergic neurons [22]. A significant decrease in TH-positive cells was observed following MPTP administration, indicating dopaminergic neuronal loss. Previous studies have also reported decreases in TH-positive cells following MPTP administration [15]. Studies using mice have shown that MPTP-induced PD-like symptoms were characterised by marked motor deficits and loss of TH-positive neurons [23]. In addition, decreased TH expression levels have been reported in the nigrostriatal dopaminergic neurons of PD patients [24]. In the present study, AZD treatment at all the three tested doses significantly improved TH-positive cell counts, as well as TH protein expressions dose-dependently, with 50 mg/kg AZD exhibiting the highest effects.

It has been shown that injection of MPTP in mice caused dopaminergic neuronal cell death in SNc via activation of the apoptotic pathway [15]. The Bcl-2 family proteins are the key regulators of cellular apoptosis and are involved in MPP+ and MPTP-induced apoptotic cell death [12]. In particular, Bcl-2 protein regulates and preserves the integrity of the mitochondrial membrane. It prevents the release of Cyt-C to the cytosol and suppresses mitochondria-mediated initiation of cell damage. In contrast, Bax, is a pro-apoptotic protein that promotes the release of Cyt-C from mitochondria [25]. In turn, Cyt-C activates caspase-3 which eventually initiates the activation of the caspase cascade.

Neuronal degeneration results in decreases in the levels of the anti-apoptotic protein Bcl-2, and an increase in the level of the pro-apoptotic protein Bax [26]. In this study, MPTP administration increased Bax in the mitochondria, while it decreased mitochondrial Bcl-2 levels. Increased Cyt-C in the cytosol reflects increased Bax levels. Cleaved Caspase-3 level is a major marker of cellular apoptosis. In this study, it was observed that MPTP administration caused

marked increase in cleaved caspase-3 level in the cytosol. This may have led to the activation of the apoptotic pathway, leading to neuronal loss in SNpc. Interestingly, AZD treatment caused noticeable decrease in levels of cleaved caspase-3, and also caused significant reversal of events that lead to apoptosis. These events include improved membrane integrity which was reflected in restoration of the Bcl-2/Bax ratio, and reduced the release of Cyt-C from mitochondria, thereby preventing the loss of dopaminergic neurons.

Oxidative stress and neuroinflammation are major contributors to MPTP-induced apoptotic death of dopaminergic neurons [27]. The MPTP-induced ROS disrupt mitochondrial membrane integrity, leading to raised levels of cyt-C in the cytosol and activation of caspase-3 [12]. In the present investigation, MPTP-administration increased the levels of ROS and MDA, indicating oxidative stress in the brain. One reliable index of lipid peroxidation is MDA, which also indicates the degree of oxidative stress [28]. Moreover, MPTP reduced the total glutathione content, and lowered the GSH:GSSG ratio. Altered GSH:GSSG ratio indicates redox imbalance, a phenomenon which has been reported in PD [29]. Reduced glutathione (GSH) is well documented as one of the major antioxidants in the brain. Other studies have also reported reduced GSH levels in the brain tissues of MPTP-induced PD model [30,31]. The observed MPTP-induced decrease in GSH is thought to be an early event in PD which triggers a cascade of molecular events, eventually leading to oxidative stress in the dopaminergic system [32].

The MPTP administration also downregulated the expressions of DJ-1, Nrf2, HO-1 and NQO1. Heme oxygenase-1 (HO-1) and NAD(P)H-ubiquinone oxidoreductase-1 (NQO1) are enzymes involved in GSH synthesis [33]. Azadiradione (AZD) treatment significantly countered oxidative stress status by reducing ROS and lipid peroxidation levels as indicated in reduced MDA content. Azadiradione also significantly improved GSH levels, and increased GSH: GSSG ratio. The AZD-induced upregulations of the expressions of SOD1, HO-1 and NQO might have also contributed to the enhancement in GSH levels, and aided in the reduction of MPTP-induced oxidative stress. The restoration of the redox balance and enhancement of antioxidant status suggest that AZD was effective in combating MPTP-induced PD.

Cytokines (inflammatory mediators) secreted by the neuronal cells are key contributors to neuronal degeneration in PD [34]. Microglial cells are the chief cellular sources of these inflammatory meditators [6]. In this study, the MPTP-treated mice presented elevated levels of iNOS, IL-1β, IL-6 and TNF-α. Increased levels of cytokine IL-1β have been reported to stimulate the production of cytokines such as TNF-α and IL-6 in microglia and astrocytes [35]. The inflammatory cytokines are known to cause activation of iNOS. High levels of iNOS enhance the concentrations of NO and superoxide radicals, which either directly or indirectly result in loss of dopaminergic neurons in PD [36]. Studies in PD patients have also demonstrated elevated cytokine levels in the brain and in CSF [37].

Experimental and clinical data have demonstrated that persistent microglial activation contributes to loss of dopaminergic neurons [38]. Azadiradione-mediated decrease in IL-1β could have, at least in part, contributed to the decreases in TNF-α and IL-6 levels. Elevated GFAP level was observed following MPTP-administration, indicating increased ROS and neuro-inflammation. The AZD-mediated significant decreases in expressions of GFAP, IL-1β, IL-6 and TNF-α suggest reduced microglial activation. These findings reflect the potent anti-inflammatory effects of AZD which could aid in the prevention of PD.

CONCLUSION

AZD-mediated reductions in inflammatory responses via down-regulation of expressions of iNOS, inflammatory cytokines and GFAP are indicative of the potential of AZD in mitigating PD symptoms. AZD-induced enhancement of antioxidant levels, decrease in oxidative stress, and reduction in α-synucelin levels contribute to increase in the population of TH-positive cells and reduction in dopaminergic neuronal degeneration. Thus, AZD may be developed into a drug for the prevention of PD-related symptoms. However, further studies are needed to unravel the full mechanisms involved in the protective effect of AZD.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this research study was

executed by the author(s) – Tao Jin, Xuemei Cao, Zongwen Gao, Xue-Qin Yan and all liabilities pertaining to claims relating to the content of this article will be borne by them. All authors equally contributed to this study. Tao Jin and Xue-Qin Yan designed this study, collected and analysed the data, and also prepared the manuscript. Xuemei Cao and Zongwen Gao contributed in the experimental works and also in statistical study.

Open Access

This is an Open Access article that uses a funding model which does not charge readers or their institutions for access and distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0) and the Budapest Open Access Initiative (http://www.budapestopenaccessinitiative.org/read), which permit unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited.

REFERENCES

1. *Lang AE, Lozano AM. Parkinson's Disease. N Engl J Med 1998; 339: 1044-1053.*
2. *Hong Z, Shi M, Chung KA, Quinn JF, Peskind ER, Galasko D, Jankovic J, Zabetian CP, Leverenz JB, Baird G, et al. DJ-1 and alpha-synuclein in human cerebrospinal fluid as biomarkers of Parkinson's disease. Brain 2010; 133: 713-726.*
3. *Bourdenx M, Bezard E, Dehay B. Lysosomes and alpha synuclein form a dangerous duet leading to neuronal cell death. Front Neuroanat 2014; 8: 83.25177278.*
4. *Klockgether T. Parkinson's disease: clinical aspects. Cell Tissue Res 2004; 318: 115-120.*
5. *Wu Y, Li X, Zhu JX, Xie W, Le W, Fan Z, Jankovic J, Pan T. Resveratrol-activated AMPK/SIRT1/autophagy in cellular models of Parkinson's disease. Neurosignals 2011; 19: 163-174.*
6. *Smith JA, Das A, Ray SK and Banik NL. Role of pro-inflammatory cytokines released from microglia in neurodegenerative diseases. Brain Res Bull 2012; 87: 10-20.*
7. *Mogi M, Harada M, Riederer P, Narabayashi H, Fujita K, Nagatsu T. Tumor necrosis factor-a (TNF-a) increases both in the brain and in the cerebrospinal fluid from parkinsonian patients. Neurosci Lett 1994; 165: 208-210.*
8. *Przedborski S, Jackson-Lewis V, Naini AB, Jakowec M, Petzinger G, Miller R, Akram M. The parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): a technical review of its utility and safety. J Neurochem 2001; 76: 1265-1274.*
9. *Smeyne RJ, Jackson-Lewis V. The MPTP model of Parkinson's disease. Mol Brain Res 2005; 134: 57-66.*
10. *Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. Neuron 2003; 39: 889-909.*
11. *Chen XC, Chen Y, Zhu YG, Fang F, Chen LM. Protective effect of ginsenoside Rg1 against MPTP-induced apoptosis in mouse substantia nigra neurons. Acta Pharmacol Sin 2002; 23: 829-834.*
12. *Adachi S, Cross AR, Babior BM, Gottlieb RA. Bcl-2 and the outer mitochondrial membrane in the inactivation of cytochrome c during Fas-mediated apoptosis. J Biol Chem 1997; 272: 21878.*
13. *Singh N, Sastry MS. Antimicrobial activity of Neem oil. Ind J Pharmacol 1997; 13: 102-106.*
14. *Garber JC. Committee for the update of the guide for the care and use of laboratory animals. In: Guide for the care and use of laboratory animals. 8th ed. National Academy of Sciences, USA; 2011.*
15. *Li H, Park G, Bae N, Kim J, Oh MS, Yang HO. Anti-apoptotic effect of modified Chunsimyeolda-tang, a traditional Korean herbal formula, on MPTP-induced neuronal cell death in a Parkinson's disease mouse model. J Ethnopharmacol 2015; 176: 336-344.*
16. *Uversky VN. Neurotoxicant-induced animal models of Parkinson's disease: understanding the role of rotenone, maneb and paraquat in neurodegeneration. Cell Tissue Res 2004; 318: 225-241.*
17. *Spillantini M, Schmidt M, Lee V, Trojanowski J, Jakes R, Goedert M. α-Synuclein in Lewy bodies. Nature 1997; 388: 839-840.*
18. *Javitch JA, D'Amato RJ, Strittmatter SM, Snyder SH. Parkinsonism-inducing neurotoxin, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: uptake of the metabolite Nmethyl- 4-phenylpyridine by dopamine neurons explains selective toxicity. Proc Natl Acad Sci USA 1985; 82: 2173-2177.*
19. *Segura AJ, Kostrzewa RM. Neurotoxins and neurotoxic species implicated in neurodegeneration. Neurotox Res 2004; 6: 615-630.*
20. *Du Y, Dodel RC, Bales KR, Jemmerson R, Hamilton-Byrd E, Paul SM. Involvement of a caspase-3-like cysteine protease in 1-methyl-4-phenylpyridinium-mediated apoptosis of cultured cerebellar granule neurons. J Neurochem 1997; 69: 1382-1388.*
21. *Daubner SC, Le T, Wang S. Tyrosine hydroxylase and regulation of dopa- mine synthesis. Arch Biochem Biophys. 2011; 508: 1-12.*
22. *Fukuda T, Takahashi J, Tanaka J. Tyrosine hydroxylase-immunoreactive neurons are decreased in number in the cerebral cortex of Parkinson's disease. Neuropathology 1999; 19: 10-13.*
23. *Inden M, Kitamura Y, Abe M, Tamaki A, Takata K, Taniguchi T. Parkinsonian rotenone mouse model: re-evaluation of long-term administration of rotenone in C57BL/6 mice. Biol Pharm Bull 2011; 34: 92-96.*
24. *Joyce JN, Smutzer G, Whitty CJ, Myers A, Bannon MJ. Differential modification of dopamine transporter and tyrosine hydroxylase mRNAs in midbrain of subjects with Parkinson's, Alzheimer's with parkinsonism, and Alzheimer's disease. Mov Disord 1997; 12: 885-897.*

25. Pak YMK. Reviews: mitochondria: the secret chamber of therapeutic targets for age-associated degenerative diseases. Biomol Ther 2010; 18: 235-245.
26. Reed JC. Proapoptotic multidomain Bcl-2/Bax-family proteins: mechanisms, physiological roles, and therapeutic opportunities. Cell Death Differ 2006; 13: 1378-1386.
27. Reynolds A, Laurie C, Mosley RL, Gendelman HE. Oxidative stress and the pathogenesis of neurodegenerative disorders. Int Rev Neurobiol 2007; 82: 297-325.
28. Jose JK, Kuttan G, Kuttan R. Antitumour activity of Emblica officinalis. J Ethnopharmacol 2001; 75: 65-69.
29. Jia P, Xu YJ, Zhang ZL, Li K, Li B, Zhang W, Yang H. Ferric ion could facilitate osteoclast differentiation and bone resorption through the production of reactive oxygen species. J Ortho Res 2012; 30: 1843-1852.
30. Munoz A, Rey P, Guerra MJ, Mendez-Alvarez E, Soto-Otero R, Labandeira-Garcia JL. Reduction of dopaminergic degeneration and oxidative stress by inhibition of angiotensin converting enzyme in a MPTP model of Parkinsonism. Neuropharmacology 2006; 51: 112-120.
31. Kavitha M, Nataraj J, Essa MM, Memon MA, Manivasagam T. Mangiferin attenuates MPTP induced dopaminergic neurodegeneration and improves motor impairment, redox balance and Bcl-2/Bax expression in experimental Parkinson's disease mice. Chem Biol Interact 2013; 206: 239-247.
32. Wullner U, Löschmann PA, Schulz JB, Schmid A, Dringen R, Eblen F, Turski L, Klockgether T. Glutathione depletion potentiates MPTP and MPP+ toxicity in nigral dopaminergic neurons. Neuroreport 1996; 7: 921-923.
33. Ansari N, Khodagholi F, Amini M. 2-Ethoxy-4,5-diphenyl-1,3-oxazine-6-one activates the Nrf2/HO-1 axis and protects against oxidative stress-induced neuronal death. Eur J Pharmacol 2011; 658: 84-90.
34. Jenner P. Oxidative stress in Parkinson's disease. Ann Neurol 2003;53: S26-36 [discussion S36-28].
35. Allan SM, Tyrrell PJ, Rothwell NJ. Interleukin-1 and neuronal injury. Nat Rev Immunol 2005; 5: 629-640.
36. Koppula S, Kumar H, Kim IS, Choi DK. Reactive oxygen species and inhibitors of inflammatory enzymes, NADPH oxidase, and iNOS in experimental models of Parkinson's disease. Mediat Inflamm 2012; 2012: 823902.
37. Koprich JB, Reske-Nielsen C, Mithal P, Isacson O. Neuroinflammation mediated by IL-1beta increases susceptibility of dopamine neurons to degeneration in an animal model of Parkinson's disease. J Neuroinflammation 2008; 5: 8.
38. Kohutnicka M, Lewandowska E, Kurkowska-Jastrzebska I, Członkowski A, Członkowska A. Microglial and astrocytic involvement in a murine model of Parkinson's disease induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Immunopharmacology 1998; 39: 167-180.