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Research Article

Resveratrol Attenuates Inflammatory Responses through Inhibiting TLR4/Myd88 Signalling Pathway in a Mouse Model of Parkinson's Disease

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Abstract

Objective: The present study aims to show the effects of Resveratrol (RV) on behavioural recovery in a mouse model of Parkinson's disease (PD) model through anti-inflammatory mechanisms.

Methods: For our Parkinson's disease model we injected mice with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). To track the behavioural performance of the mice we used pole test and traction tests. The levels of expression of Tyrosine Hydroxylase (TH) and a-synuclein (a-syn) in the substantia nigra of the mice were determined by immunohistochemistry. The expression of Toll-like receptor 4 (TLR4) and MyD88 protein in the substantia nigra pars compacta were detected using western blotting, and the levels of proinflammatory cytokines tumour necrosis factor-alpha (TNF-α), Interleukin (IL)-1β, and IL-6 were determined by ELISA.

Results: Treatment with RV resulted in improved motor function of PD mice. Such behavioural effects were accompanied with lower levels of TLR4 and MyD88 protein expression, and reduced concentration of proinflammatory cytokines in the substantia nigra pars compacta. Finally, RV treatment increased the level of expression of TH while decreasing the expression level of a-syn in the substantia nigra.

Conclusion: These findings indicate that RV can attenuate inflammatory responses by suppressing the TKR4/MyD88 signalling pathway, which coincides with better behavioural performance.

Significance: The improved behavioural function and decreased activity of the inflammatory pathways may open the ways for better treatments for people with PD.

Keywords: Parkinson's Disease; Resveratrol; TLR4; Inflammatory

Introduction

Parkinson's Disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease [1]. PD is pathologically characterised by loss of dopaminergic neurons in the substantia nigra and is clinically characterised by shaking, muscle rigidity, bradykinesia, and other motor and cognitive signs and symptoms [2]. It is estimated that 1-2 per thousand of the world's population are affected by the deleterious symptoms of this disease [3].

Unfortunately, however, the exact pathophysiology of PD, to date remains unclear. Currently, studies indicate that inflammation is involved in the aetiology of PD [4]. In post-mortem studies, it has been found that various typical pro-inflammatory cytokines in the brains of PD patients are increased, including tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β and IL-6 [5]. Treatment with non-steroidal anti-inflammatory drugs can reduce the risk of PD development [6].

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Toll-like receptor 4 (TLR4)/MyD88 axis is an important signalling pathway during inflammation; the role of TLR4 in PD neuro inflammation has attracted attention in recent years [7]. Previous studies have shown that the expression level of TLR4 in the peripheral blood of PD patients is higher than that of healthy volunteers. Importantly, the level of expression of TLR4 is closely related to both the aetiology and prognosis of PD and the response to pharmacological treatments [8]. This shows that the TLR4/MyD88 axis does play an important role in the pathogenesis of PD.

Resveratrol (RV) is a widely known polyphenol compound, found in grapes, mulberries, peanuts, rhubarb, and various other plants. At molecular level, it targets adenosine monophosphate kinase, NF-κB, inflammatory cytokines, antioxidant enzymes, and metabolic and cellular processes such as gluconeogenesis, lipid metabolism, mitochondrial biogenesis, angiogenesis, and apoptosis [9]. RV exhibits antioxidant, anti-inflammatory, anti-cancer, anti-neurodegenerative, and estrogenic properties. A recent study reports that RV can inhibit the expression of Toll-like receptors and pro-inflammatory genes [10]. In the present study, the expression of TLR4 and MyD88 and the effects of RV on inflammatory responses are evaluated in an MPTP-induced mouse model of PD. In addition, the possible regulatory action on the TLR4/MyD88 signalling pathway is assessed.

Materials and Methods

Forty Male, eight-week-old, C57BL/6N mice obtained from Weitong Lihua laboratory animal technology Co. Ltd. in Zhejiang, China, were maintained in a standard experimental animal room at a constant temperature (22 ± 1°C), relative humidity (60%), a strict 12 h/12 h light-dark cycle, with ad-libitum access to water and food. All experimental procedures were approved by the Ethics Committee of The First Affiliated Hospital of Xinxiang Medical University (code : NO2019027) and performed according to the guidelines for the Care and Use of Laboratory

Animals. The mice were randomly divided into four groups: Saline control, RV, MPTP, MPTP+RV (n = 10 in each group). Mice in the MPTP+RV group received an intraperitoneal injection of MPTP (M832929, Macleans Biochemical Technology Co. Ltd., Shanghai, China) at 30 mg/kg daily for seven days followed by RV treatment 3 weeks.

RV (R5010, Sigma-Aldrich, St Louis, MO, USA) was dissolved in saline and administered via oral gavage at 30mg/kg daily for three weeks. Mice in the saline control group received the same volume of saline throughout the study. The mice in the RV group received the same volume of saline intraperitoneal injection daily for seven days, followed by RV gavages for 3 weeks. The mice in the MPTP group received an intraperitoneal injection of MPTP at 30mg/kg daily for seven days, which was followed by the same volume of saline gavage during the treatment days of RV group. The mice were euthanised at the end of the behavioural tests and the tissues were collected for histological examinations. All of the mice underwent behavioural tests. Six mice from each group were randomly selected for ELISA and western blot analysis, with the other four mice used for histological assessments (Figure 1).

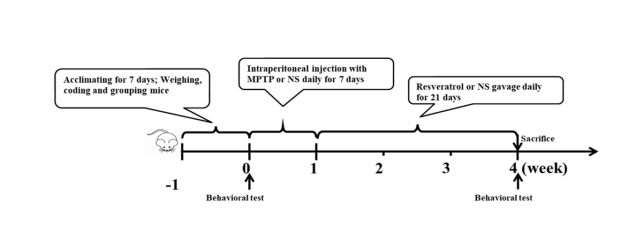

Figure 1

Behavioural Test

Pole test was performed (as previously described [11]) before the MPTP intraperitoneal injections, on day seven of the MPTP intraperitoneal injections and day 21 of the RV treatment. Briefly, each mouse was positioned head upwards on the small ball at the top of a wooden pole; the time taken to turn the head and climb down the pole was recorded. The pole was 0.8 cm in diameter, 60 cm in height and rough-surfaced, with a small ball (0.5 cm in diameter) on the top. The test was repeated three times, and the mean of the head turning and descent time was used for analyses.

A traction test was performed on a horizontal wire (1.5 mm in diameter), one hour after the pole test respectively, at 30cm from the ground. The forepaws of the mice were placed on the wire and the times before landing were scored according to following criteria: 0: 0~10 s; 1: 11~20 s; 2: ~30 s; 3: 31~40 s; 4: 41~50 s; 5: 51~60 s; 6: over 60 s [9].

Western Blot

The midbrain tissues were homogenised in radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with 1% phenylmethanesulfonyl fluoride and 1% protease inhibitor cocktail (all Beyotime) on ice. The homogenate was centrifuged at 12000 r/min at 4°C for 15 minutes and the supernatant was collected. Nuclear proteins were extracted using a nuclear and cytoplasmic protein extraction kit (Beyotime) in accordance with the manufacturer's protocol. The protein concentration was determined using a BCA protein assay kit (Beyotime). Equal amounts of protein samples (each 40 ug) were separated using 10% sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking in 5% skimmed milk, the membranes were incubated with primary antibodies at 4°C overnight. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit (bs-40295G-HRP, Bioss Biochemical Technology Co. Ltd., Beijing, China) at 37°C for 40 minutes. Protein blots were visualised with enhanced chemiluminescence (Millipore, WBKLS0100, USA) and quantified using Image-Pro Plus software for analysis. β-actin was used as a loading control. The primary antibodies used were as follows: anti-TLR4 antibody (1:500, bs-20594R), anti-Myd88 antibody (1:500, bs-1047R), and β-actin (1:1000, bs-0061R).

Measurement of Proinflammatory Cytokines

The brain was harvested immediately after the sacrifice of the mice. The ventral midbrain, containing the substantia nigra pars compacta (SNpc), was isolated and homogenised in cooled PBS on ice. The homogenate was centrifuged at 400g for 15 minutes and the supernatant was collected for measurement of proinflammatory cytokines. The levels of TNF-α, IL-1β, and IL-6 in the ventral midbrain were determined using commercial ELISA kits (Multisciences, Beijing, China) according to manufacturer's protocols.

Immunohistochemistry

The midbrain tissues were immersion fixed in 4% paraformaldehyde at 4°C for 24 hours, embedded in paraffin, and cut into 5-μm-sections. The sections were deparaffinised in xylene, hydrated using absolute ethanol, 85% alcohol and 75% alcohol successively, and boiled in sodium citrate antigen retrieval solution for 15 minutes. The sections were then incubated with endogenous peroxidase blocking solution for 25 minutes at room temperature. After a wash step, the sections were blocked with 3% H2O2 for 25 minutes at room temperature, after which the sections were blocked with 3% BSA for 30 minutes. The sections were incubated in anti-Tyrosine Hydroxylase (TH) antibody (1:750, rabbit polyclonal, ab112, Abcam, Cambridge, UK) and a-syn antibody (1:200, rabbit polyclonal, ab138501, Abcam, Cambridge, UK) at 4°C overnight. After a wash step, the sections were incubated in HRP-labelled goat anti-rabbit (1:200, Beyotime) at 37°C for 50 minutes. The next step was DAB color development, we controlled the color development time by checking the tissue under the microscope, the positive signal is brownish yellow and once it develops, the color development is terminated by rinsing with the tap water. Next, we counterstain with hematoxylin for about 1 min, rinse with tap water resulting in the bluing of the hematoxylin, then the water is rinsed. Finally, dehydration and and cover slipping is done. The tissues were then examined under light microscope. Images were acquired and analysed.

Statistical Analysis

Graph Pad Prism 8.0 software was used to perform statistical analysis on the data; data are expressed as: mean (x) ± standard deviation (s). Statistical analysis was carried out using one-way analysis of variance (ANOVA). $P < 0.05$ indicates that the difference is statistically significant.

Results

RV Improved MPTP-Induced Dyskinesia in Mice

To assess the effects of RV treatment on motor function, pole and traction tests were run in the present study. The pole and traction tests were run before the experiment. (Baseline), then after the development of disease model, and finally after the treatment. The baseline performance was the similar among the groups. After the disease modelling, the results of the pole and traction tests showed that MPTP injection significantly increased the head turning time and descent time, while decreased limb movement score compared to the sham group (Figures 2A-2C). Interestingly, RV treatment significantly decreased the head turning time and descent time and also the increased limb movements score, indicating an improvement in total locomotor activity and limb movements.

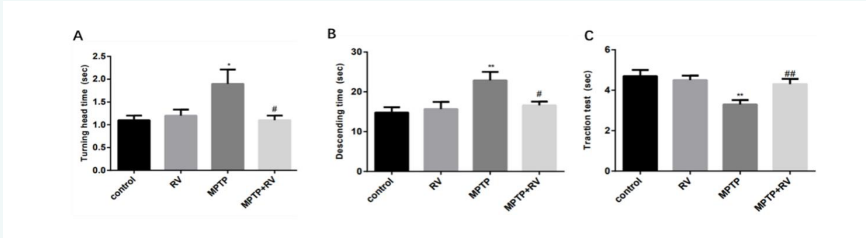

Figure 2 illustrates the RV improved MPTP-induced dyskinesia in mice, and the effect on pole testing (A,B) and traction testing (C) at three weeks after the first RV treatment. $^{*}P < 0.05$ *vs.* control; $^{**}P < 0.01$ *vs.* control; $^{\#}P < 0.05$ *vs.* MPTP; $^{\#\#}P < 0.01$ *vs.* MPTP, n = 10 mice in each group..

RV Ameliorates Pathological Changes in the Substantia Nigra in the Midbrain of PD Mice

As illustrated in (Figure 3), lower expression of TH positivity was observed in the substantia nigra of the MPTP-injected mice versus the control mice, (Figure 3), indicating the successful development of the disease model. RV treatment provided significant improvement from MPTP-induced reduction of TH signalling in the midbrain. High expression of a-syn positive cells was observed in substantia nigra of the MPTP-injected mice versus the control mice, (Figure 4). RV treatment provides important improvement from MPTP-induced increase of a-syn in the midbrain.

RV Attenuates MPTP-Induced Inflammatory Responses

The anti-inflammatory effect of RV was further confirmed in the ventral midbrain. As shown in (Figure 4), MPTP significantly increased the secretion of pro-inflammatory cytokines TNF-, IL-1, and IL-6, while RV treatment markedly suppressed the secretion of those cytokines.

RV Inhibited MPTP-Induced TLR4/Myd88 Factor Activation

Using western blotting, activation of the TLR4/MyD88 signalling pathway in the ventral midbrain was evaluated and it was found that protein expression levels of TLR4 and its downstream

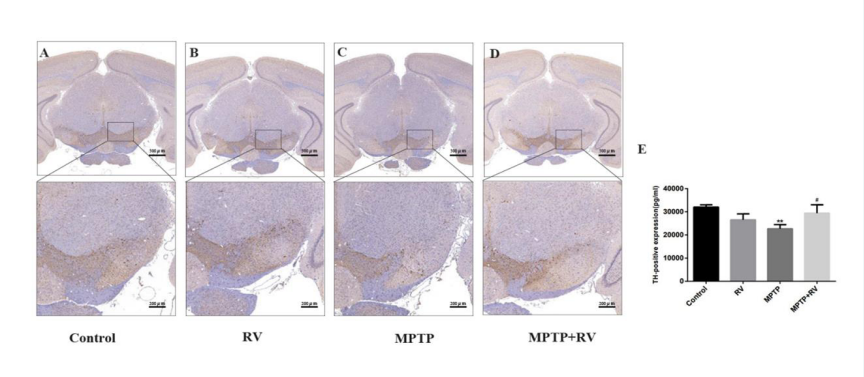

Figure 3 RV increased TH expression in the ventral midbrain of MPTP-induced PD mice.
It representative pictures of TH staining in substantia nigra of mice in each group; A,B,C and D: show the substantia nigra of each group of mice under low power microscope. Scale = 500 μm; below is the enlarged view of frame A,B,C and D. Scale bar = 200 μm, n = 4. E: TH positive expression results, $^{*}P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$ *vs.* MPTP, n = 4 mice in each group..

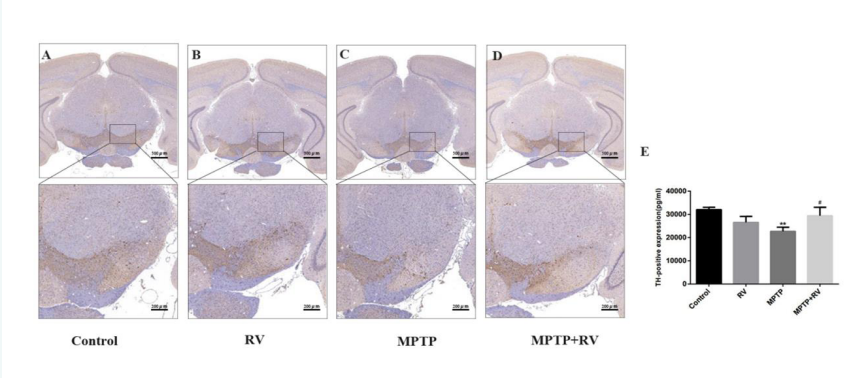

Figure 4 RV attenuated a-syn expression in the ventral midbrain of MPTP-induced PD mice.
Representative pictures of a-syn staining in substantia nigra of mice in each group; A,B,C and D: show the substantia nigra of each group of mice under low power microscope. Scale = 500 μ m; below is the enlarged view of frame A,B C and D. Scale bar = 100 μm, n = 4. E: A-syn positive expression results, $^{*}P < 0.05$ *vs.* control; $^{**}P < 0.01$ *vs.* control; $^{\#}P < 0.05$ *vs.* MPTP, n = 4 mice in each group.

protein MyD88 were upregulated after MPTP injection. Nevertheless, RV treatment markedly downregulated the expression levels of TLR4 and MyD88 (Figure 5). Protein blots were visualized with enhanced chemiluminescence and quantified using Image-Pro Plus software for analysis. β -actin was used as a loading control.

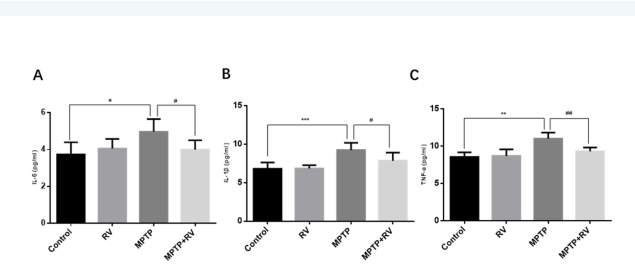

Figure 5 RV attenuated MPTP-induced inflammatory responses in the ventral midbrain. The amounts of TNF- (A), IL-1 (B), and IL-6 (C) in the ventral midbrain of mice.
$^{*}P < 0.05$ vs. control; $^{***}P < 0.001$ vs. control; $^{\#}P < 0.05$ vs. MPTP, n = 6 mice in each group.

Discussion

This study investigates the mechanism of RV's anti-PD effects from the perspective of inflammation. Firstly, the success of the disease model was verified through mouse behavioural assessment and midbrain parenchymal immunohistochemistry. ELISA results showed that IL-6, IL-1β and TNF-α in mice after modelling were significantly higher than those in sham control mice. Secondly, it was found that RV treatment can improve the motor function of PD mice and reduce the expression of TLR4, MyD88, IL-6, IL-1β and TNF-α in the midbrain of PD mice. This research shows that the anti-neuro-inflammatory effects of RV may be related to the inhibition of the TLR4/MyD88 signalling pathway.

TH is a rate-limiting enzyme in the convertion of tyrosine to L-DOPA. The characteristic pathological feature of PD is the presence of cytoplasmic accumulation in Lewy Bodies (LBs) and Lewy neurites; a-syn is one of the main intracellular protein aggregation in LBs. Various sources of emerging evidence suggest that the a-syn gene and protein aggregation are strongly associated with occurrence and development of PD [12], with the increased a-syn expression playing a critical role in the pathophysiology process of PD. Downregulation of TH expression and upregulation of a-syn leads to a reduction in dopamine production and causes PD [13]. The pole test and the traction test can be used to evaluate the motor function of PD mice [11].

The present study demonstrates that RV treatment increases the expression of TH-positive cells and decreases the expression of a-syn in the substantia nigra of PD mice, which indicates that treatment of RV may mitigate the MPTP-induced loss of TH and the increase in a-syn. Due to the dopaminergic dysfunction in the brain, MPTP also causes functional deficits in animals. In the present study, RV treatment counteracted MPTP-induced reduction of locomotor activity and dysfunction of limb movements. These findings suggest that RV exhibits a neuroprotective effect against MPTP-induced PD in mice.

Previous studies have shown that the pathogenesis of neurodegenerative diseases is characterized by oxidative stress, cytotoxicity, inflammation, and abnormal protein accumulation in the nervous system, among which neuroinflammation is thought to play a key role in the occurrence and progression of PD [14]. In 1988, activation of substantia nigra microglia was observed in PD patients, which was the first report of inflammation in the CNS [15]. Although the role of inflammation in the occurrence and development of PD has not been fully elucidated, there is growing evidence that inflammation is related to PD. Various studies have shown that RV has anti-oxidative, anti-inflammatory, anti-tumour, and other pharmacological effects, for examples it has been shown to protect the heart [16], acting as an anti-diabetic [17], preventing cancer [18], and providing neuroprotection [19,20]. The anti-inflammatory action of RV has been comprehensively studied in previous research [21,22]. The present study finds that RV improves motor function of PD mice, and additionally could reduce MPTP-induced secretion of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6. These data demonstrate that the anti-inflammatory action of RV might be associated with its anti-PD activity.

Toll-like receptors are pattern recognition receptors in the body's primary immune recognition system, which can recognise molecular patterns related to different pathogens, thereby initiating downstream related signal transduction pathways [23]. The HMGB1/TLR4 axis is a key pro-inflammatory signalling pathway which plays an important role in the promotion of neuritis [24]. In PD, clinical studies have found that, compared to healthy volunteers, the expression of HMGB1/TLR4 axis protein in PD patients is significantly up-regulated, and the expression of its downstream factors MyD88, NF-κB, and TNF-α are significantly up-regulated [6]. The studies illustrate the fact that the MPTP toxicity of TLR4-deficient mice is weaker than normal, and MPTP toxicity is related to the decreased activation of microglia in the substantia nigra, which indicates that the death of dopaminergic cells is, at least to some extent, TLR4-dependent [25-27].

In animal experiments, rotenone treatment in TLR4 knockout mice showed less intestinal inflammation [7]. Rotenone results in lower intestinal and motor dysfunction, brain nerve inflammation, and neurodegeneration [7]. Therefore, inhibiting this signalling pathway (TLR4) is helpful to the treatment of PD. The present study finds that RV can improve the inflammatory response in PD mice, but the mechanism for this remains unclear. The effect of RV on TLR4/MyD88 axis has been demonstrated in the studies on other tissues [28-30].

To explore the possible mechanism of RV reducing inflammation in PD mice, this study looked at the expression of TLR4 and MyD88 in the substantia nigra of the ventral midbrain of PD mice. In line with these studies, the results of the present study also finds that treatment with RV markedly downregulates the protein expression levels of TLR4 and MyD88. These results suggest that suppressing the activation of the TLR4/MyD88 axis might form part of the neuroprotection of RV against PD.

In conclusion, the findings of this study show that RV can reduce neuroinflammation in PD mice. This anti-neuroinflammatory effect of RV may be related to the inhibition of the activation of TLR4/MyD88 signalling pathway.

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