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Original Article

Nervonic acid alleviates MPTP-induced Parkinson's disease via MEK/ERK pathway

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Abstract

Nervonic acid (NA) is a primary long-chain fatty acid and has been confirmed to have neuroprotective effects in neurologic diseases. Oxidative stress and neuronal damage are the main causes of Parkinson's disease (PD). This study mainly explored whether NA is involved in regulating oxidative stress and apoptosis in MPTP-induced mouse model and MPP-induced cell model. Through behavior tests, we proved that MPTP-induced motor dysfunction in mice was recovered by NA treatment. NA can reduce MPTP-induced neuronal damage, manifested by elevated levels of TH and dopamine, as well as decreased levels of α-syn. In the in vitro model, we observed from CCK8 assay and flow cytometry that the induction of MPP markedly suppressed cell activity and enhanced cell apoptosis, but these functions were all reversed by NA. Furthermore, NA administration reversed the increase in ROS production and MDA levels induced by MPTP or MPP, as well as the decrease in SOD levels, suggesting the antioxidant properties of NA in PD. Meanwhile, we confirmed that NA can regulate oxidative stress and neuronal damage by activating the MEK/ERK pathway. Overall, we concluded that NA could alleviate MPTP-induced PD via MEK/ERK pathway.

Keywords: Parkinson's disease, Nervonic acid, Oxidative stress, ERK signaling.

1. Introduction

Parkinson's disease (PD) is an irreversible neurodegenerative disease that mainly occurs in middle-aged and elderly people over the age of 60 [1]. PD is a multifactorial disease caused by genetic, environmental and aging-related factors [2]. It has a high incidence rate and disability rate [3]. As the disease progresses, patients may experience motor symptoms such as static tremors, rigidity, and motor delay, as well as non-motor symptoms such as cognitive impairment, anxiety, and drowsiness [4]. The specific pathogenesis of PD is not yet clear, but it may be related to various potential risk factors, such as oxidative stress, cell death, and neuroinflammation. The loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) is the major pathological characteristic of PD [5]. It can initiate the formation of Lewy bodies, which consist largely of α-synuclein (α-syn) [6]. The agglomeration of α-synuclein in brains is conducive to oxidative stress and neurodegeneration [7]. Apoptosis has a vital function in neuronal death [8]. The current treatment strategy for PD can only improve symptoms and slow down disease progression, while cannot completely cure this disease. Moreover, current drugs all have certain side effects [9]. Therefore, it is crucial to deeply explore the pathogenic mechanism of PD and develop new and effective drugs.

Nervonic acid (NA) is the main active compound of Xanthoceras sorbifolia Bunge, which has various pharmacological properties [10]. NA is a primary long-chain fatty acid that is abundant in the white matter of brains. It primarily sustains the composition and functions of biofilms through sphingolipids, thereby enhancing cell vitality [11]. Research efforts have shown that NA is related to brain development and is a crucial molecule for the growth and maintenance of brain and peripheral nervous tissues, playing a protective role in them [12]. NA has been proven to be closely associated with some neurological diseases [13]. The reduction of NA expression is closely related to an individual's high risk of developing mental disorders [14]. The plasma NA levels is associated with white matter dysfunction in patients with severe depression, suggesting that NA may be a prospective diagnostic biomarker [15]. Integrated metabolomics and transcriptomics have revealed that NA can restrain neuroinflammation and exert neuroprotective functions in Alzheimer's disease [16]. In previous study, it has been confirmed that NA can effectively alleviate MPTP-induced movement disorders and

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neuroinflammation in PD mice [17]. However, the specific mechanism by which NA regulates PD progression still needs further research.

Herein, we investigate whether NA can alleviate PD development by regulating apoptosis and oxidative stress in MPTP-induced PD mice and MPP-induced neuronal damage models.

2. Materials and methods

2.1. The establishment of PD mouse model

Adult male C57BL/6 mice purchased from the Baoan Maternal and Child Health Hospital, College of Medicine, Jinan University were raised in an SPF environment and allowed to eat and drink freely for a week. Animal experiments were approved by the Baoan Maternal and Child Health Hospital, College of Medicine, Jinan University. Mice were randomly divided into the control group, MPTP group, MPTP+NA (40 mg/kg) group, and MPTP+NA (80 mg/kg) group (n=5 per group). Mice were intraperitoneally injected with MPTP (20 mg/kg) for three days to induce PD symptoms. PD mice were then treated with NA (Sigma-Aldrich, St. Louis, MO, USA) through gavage. Mice in control and MPTP groups received an equal volume of PBS. After a week, mice performed behavior tests to evaluate their motor function.

2.2 Pole test

To detect movement disorders in PD mice, we prepared a long wooden pole (1 cm in diameter, 60 cm in height) covered with gauze for testing. In addition, at the top of the pole, we fixed a wooden ball with a diameter of 2 cm. During the testing, mice were placed on wooden balls and then crawled under the poles. The total time required for the mouse to descend from the pole was recorded.

2.3 Open field test

The open field test was carried out to determine spontaneous movement of mice. The experimental box was cleaned with alcohol before tests. Mice were put in the experimental box, and then we utilized the video analyzer and EthoVisionXT12 (Noldus, the Netherlands) software to analyze the movement tracks, total distance, and the number of crossings of mice.

2.4. Rotarod test

Mice were put on the automatic rotarod bar (3 cm in diameter) for testing. Within 5 minutes, we accelerated the device to 40 revolutions per minute. The experiment was conducted three times, with an hour interval between each time. We recorded the latency of mice falling from the rod.

2.5. Immunohistochemistry assay

After mice were executed, we obtained the SNc tissues and fixed them with formalin for 24 h. Then, they were embedded in paraffin and cut into 4 um thickness sections. Sections were cultured with 0.3% H_2O_2 for 15 min without light, followed by incubating with 3% BSA for half an hour. Later, sections were cultured with the primary antibodies (Abcam, USA) at 4 °C for one night. Afterwards, sections were rinsed with PBS and cultured with the secondary antibody (Abcam, USA) for 2 h. Sections were developed with DAB and the light microscope (Olympus, Japan) was employed for observation.

2.6. Biochemical studies

Cells were incubated in a 24-well plate and treated with 10 μM of DCFH-DA for half an hour in a dark room. Cells were rinsed by PBS, and ROS production was tested by fluorescence microscope (Olympus) and analyzed by ImageJ.

The contents of LDH, SOD, and MDA in the supernatant of brain tissues or cells were determined by their corresponding kits (Jiancheng Bioengineering Institute, Nanjing) in line with user guides.

For testing the dopamine levels, the DA ELISA kit (Jiancheng Bioengineering Institute) was utilized in the brain tissues of mice in accordance with user guides.

2.7. Cell culture

The human neuroblastoma cell line SH-SY5Y (Cell Bank of the Chinese Academy of Sciences, Beijing, China) was incubated in DMEM/F12 medium with 15% FBS in a 5% CO_2 atmosphere at a temperature of 37°C. Cells were incubated with 0.5, 1, or 2 mM of MPP (Sigma-Aldrich, St. Louis, MO, USA) for 24 h.

2.8. Western blot

Cells and tissues were lysed utilizing the RIPA lysis buffer (Beyotime, Shanghai, China) and subjected to centrifugation. Then proteins were isolated by SDS-PAGE and transferred to the PVDF membranes, which were blockaded with 5% skim milk for 1 h. Next, membranes were cultured with primary antibodies (Abcam) at the temperature of 4 °C for one night. They were subsequently incubated with secondary antibodies (Abcam) for 2 h. The protein bands were visualized utilizing a chemiluminescence reagent (Invitrogen, USA) and analyzed by ImageJ.

2.9. CCK8 assay

Cell viability was tested utilizing the CCK8 kit (Biosharp, Beijing, China). Cells were put in 96-well plates and incubated at different times. After attachment, cells were treated with 0.5, 1, or 2 μM of NA. Next, 10 μL of CCK8 regent was supplemented for 2 h of incubation. The absorbance was assessed through a microplate reader (Molecular Devices, USA).

2.10. Flow cytometry

Cell apoptosis was tested through FITC Annexin V Apoptosis Detection kit (BD Biosciences). Cells were double dyed with 10 μl FITC Annexin V and 10 μl PI in dark for 30 minutes. Next, the BD FACS flow cytometry and FlowJo software were applied to measure cell apoptosis rate.

2.11. Statistical analysis

Data are presented as means ± SD from three individual repeats. Statistical analysis was performed by GraphPad Prism software (version 7.0, USA). Statistical significance was calculated using one-way ANOVA or Student's *t*-test. $P<0.05$ was considered as statistical significance.

3. Results

3.1. NA improves MPTP-induced motor dysfunction in PD mice

To validate the treatment effect of NA on PD, we established the MPTP-induced PD mouse model. The structural formula of NA is shown in Figure 1A. After modeling,

we carried out the behavioral tests for assessing the movement disorder, spontaneous movement, and coordination ability of PD mice. Through the pole test, we found that compared to the controls, the time taken for PD mice to descend from the top to the bottom of the pole increased by approximately 50%. However, NA treatment markedly reduced the descending time of PD mice in a dose-dependent manner (Figure 1B). In the open-field test, we recorded a trajectory map of mouse spontaneous movement (Figure 1C). In comparison to controls, MPTP induction notably reduced the quantity of line crossing and the total distance, while NA treatment reversed their decline (Figure 1D-E). The coordination ability of PD mice was evaluated by rotarod test, and we found that MPTP-induced mice took less latency time than control mice, while NA treatment abolished the MPTP effect (Figure 1F). Thus, we proved that NA improved MPTP-induced motor dysfunction in PD mice.

3.2. NA reduces MPTP-induced neuronal damage

The effect of NA on neuronal damage in PD mice was further explored. The pathological features of PD are the loss of dopaminergic neurons in the SNc and extensive aggregation of α-syn proteins. Tyrosine hydroxylase (TH) is a precursor of dopamine. Through IHC, we discovered that TH density in SNc of MPTP-treated mice was lower than control mice, whereas it was increased by NA administration in a dose-dependent manner (Figure 2A). The density alteration of α-syn in SNc was opposite to that of TH (Figure 2B). In addition, it was proved by western blot that TH protein levels reduced by MPTP injection were recovered by the administration of 40 or 80 mg/kg NA, whileα-syn protein levels increased by MPTP induction were declined by NA (Figure 2C). Furthermore, we also

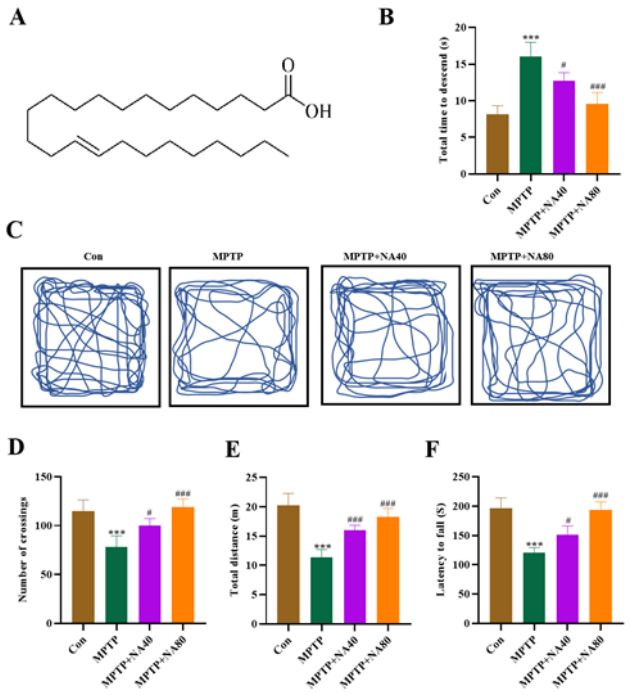

Fig. 1. NA improves MPTP-induced motor dysfunction in PD mice. (A) The structural formula of NA. (B) The time to descend the pole of mice in the pole test. (C) Movement traces of mice in the open field test. (D) The quantity of line crossings of mice in the open field test. (E) Total distance traveled of mice in the open field test. (F) The latency to fall in the rotarod test. $^{*}p<0.05$, $^{\#}p<0.05$.

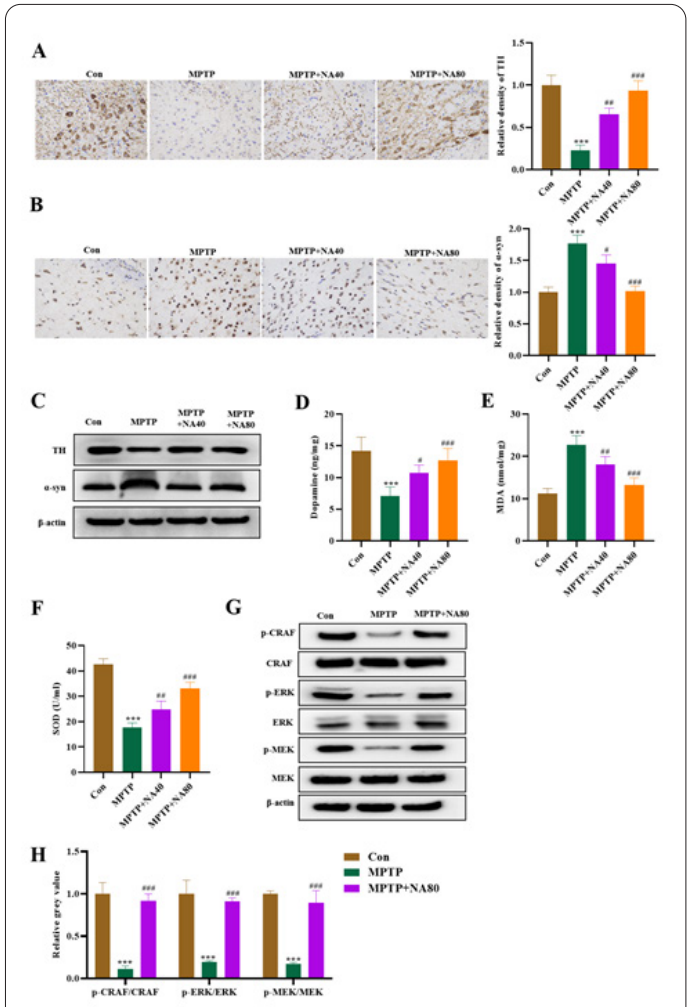

Fig. 2. NA reduces MPTP-induced neuronal damage. B) IHC analysis of the density of TH or α-syn in SNc of the control, MPTP, MPTP+NA40, and MPTP+NA80 groups. (C) Western blot outcomes of TH and α-syn levels in different groups. (D) Dopamine levels were tested in different groups. (E-F) The contents of MDA and SOD were tested in different groups. (G) Western blot outcomes of p-CRAF, CRAF, p-ERK, ERK, p-MEK, and MEK levels. $^{*}p<0.05$, $^{\#}p<0.05$.

proved that MPTP induction notably reduced dopamine content in SNc, whereas NA administration attenuated this phenomenon (Figure 2D). Oxidative stress is the main cause of dopaminergic neuron loss. Thus, we detected the effect of NA on oxidative stress. We observed that MDA content was elevated in MPTP group and this elevation could be reversed by 40 or 80 mg/kg NA (Figure 2E). Meanwhile, SOD content decreased by MPTP injection and was recovered by NA treatment (Figure 2F). The previous study has proved NA therapy in PD mice is associated with MAPK pathway [18]. Thus, we detected the level alteration of key proteins in MAPK pathway in MPTP-induced mice. The outcomes manifested that the phosphorylation of CRAF, ERK, and MEK notably declined in MPTP group, while increasing in MPTP+NA (80 mg/kg) group (Figure 2G). It suggested that NA can activate MEK/ERK pathway to regulate PD process. Overall, these outcomes proved that NA reduced MPTP-induced neuronal damage in PD mice.

3.3. NA alleviates MPP-induced apoptosis in SH-SY5Y cells

We established the PD cell model to further detect the impact of NA on neurocyte apoptosis. MPP-induced SH-

SY5Y cells are frequently utilized as the cell model of neuron injury. We utilized the CCK8 kit to determine the toxicity of MPP in SH-SY5Y. Cells were treated with 0, 0.5, 1, or 2 mM of MPP and we observed that cell viability decreased with increasing MPP concentration (Figure 3A). Additionally, we discovered the viability of MMP-induced SH-SY5Y cells decreases over time (Figure 3B). Thus, we confirmed that MPP resulted in a decrease of cell viability in a time- and dose-dependent manner. Subsequently, we proved that NA administration can abolish the suppressive function of MPP on cell viability (Figure 3C). Then, according to the analysis outcomes of flow cytometry, we found the enhancement of cell apoptosis caused through MPP induction, while this phenomenon can be reversed by NA treatment (Figure 3D). In addition, MPP-reduced Bcl-2 levels were recovered by NA treatment, and MPP-increased Bcl-2 levels were impaired by NA treatment, which further proved that NA could alleviate neurocyte apoptosis (Figure 3E). The increase in lactate production may contribute to the apoptosis of dopaminergic neurons in PD [19]. The experimental outcomes illustrated that LDH production was raised by MPP treatment in SH-SY5Y cells, however, NA administration reduced its release (Figure 3F). Next, we evaluated the impact of NA on oxidative stress. The promoting function of MPP on ROS and MDA contents was attenuated by NA administration. SOD content was restrained in MPP-treated cells and recovered in MPP plus NA-treated cells (Figure 3G-I). Finally, we discovered that MPP stimulation restrained the phosphorylation of CRAF, ERK, and MEK proteins, but NA administration reversed this effect, suggesting the activation of MEK/ERK pathway by NA (Figure 3J). Overall, we proved that NA alleviated MPP-induced apoptosis in SH-SY5Y cells.

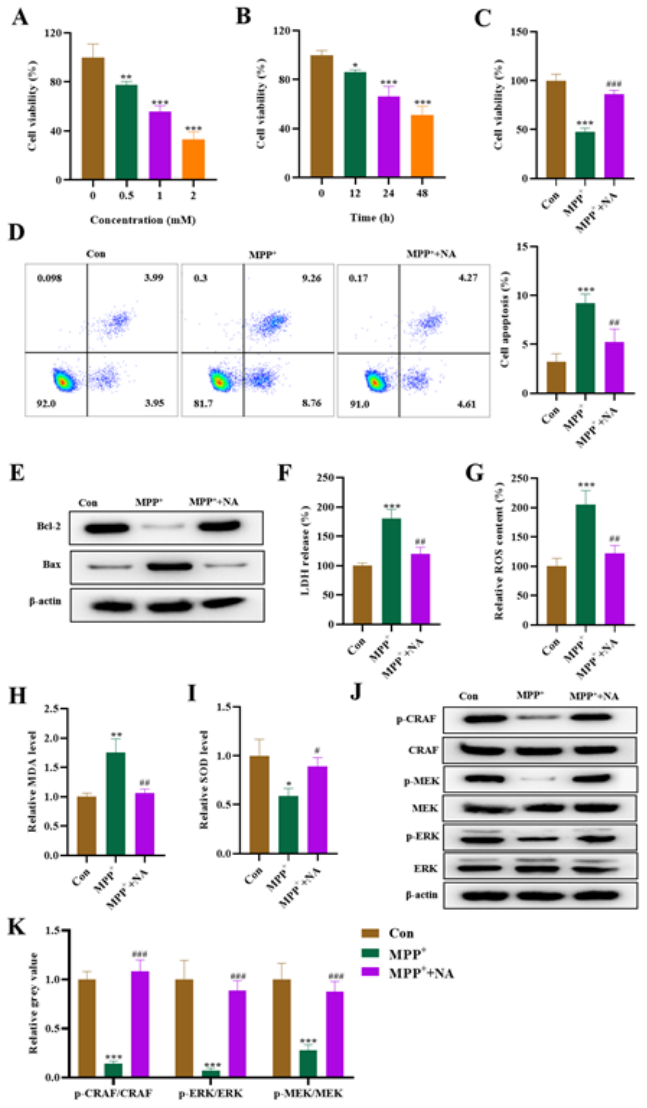

Fig. 3. NA alleviates MPP-induced apoptosis in SH-SY5Y cells. (A) CCK8 assays were implemented for testing the viability of cells treated with 0, 0.5, 1, or 2 mM NA. (B) CCK8 assays were utilized to determine the viability of 1 mM NA-treated SH-SY5Y cells at 0, 12, 24, or 48 h. (C) CCK8 assays were performed for detecting the viability of cells in the control group, MPP group, and MPP+NA group. (D) Cell apoptotic ratio was assessed through flow cytometry in different groups. (E) Western blot outcomes of Bcl-2 and Bax levels in different groups. (F) The production of LDH was tested in different cells. (G-I) The contents of ROS, MDA, and SOD were measured. (J) Western blot outcomes of p-CRAF, CRAF, p-ERK, ERK, p-MEK, and MEK levels. $^{*}p<0.05$, $^{\#}p<0.05$.

3.4. The inhibitor of MEK/ERK pathway suppresses the therapeutic effect of NA on PD process

MEK/ERK pathway was found to be activated by NA in MPTP-induced mouse model and MPP-induced cell model, thus we injected the U0126 (pathway inhibitor) into PD mice to determine whether NA alleviated PD process through MEK/ERK pathway. We discovered that TH density and protein level in SNc were increased by NA administration in MPTP mice, however, U0126 injection abolished NA effect (Figure 4A&C). On the contrary, α-syn density and protein level decreased by NA was recovered by U0126 (Figure 4B-C). Then, we further proved that NA administration elevated dopamine and SOD levels but reduced MDA levels in MPTP mice, while U0126 treatment counteracted these effects (Figure 4D-F). Overall, after U0126 injection, the therapeutic effect of NA on PD was notably restrained, confirming NA-regulated PD through the MEK/ERK pathway.

3.5. NA inhibits MPP-induced neuronal apoptosis and oxidative stress through the MEK/ERK pathway

We further examined the impacts of U0126 on the MPP-induced neurocyte injury. We found that under the condition of MPP treatment, the elevation in cell viability and the decline in cell apoptosis caused by NA treatment were reversed via U0126 treatment (Figure 5A-B). Similarly, in western blot analysis, we observed a decrease in Bcl-2 levels and an increase in Bax levels in MPP+NA+U0126 cells compared with MPP+NA cells (Figure 5C). Furthermore, we discovered that NA therapy suppressed LDH, ROS, and MDA contents in MPP^{+} cells, but elevated SOD content. However, U0126 treatment reversed all effects of NA on their contents (Figure 5D-G). Therefore, we believed that NA inhibited MPP-induced neuronal apoptosis and oxidative stress through the MEK/ERK pathway.

4. Discussion

At present, there is no specific drug to completely cure PD [20]. MPTP is a neurotoxic pollutant, and MPTP-induced mouse model has frequently been one of the most widely utilized mouse models in PD research [21]. After entering the brain, MPTP can be converted into a toxic metabolite MPP to damage dopaminergic neurons [22]. Therefore, we utilized MPTP to construct a PD mouse

model and utilized MPP-induced SH-SY5Y cells as a neuronal injury model. Motor dysfunction is a clinical symptom of PD [23]. We found through a series of behavioral tests that under the stimulation of MPTP, mice exhibited notable motor dysfunction, indicating the success of PD modeling. NA is a long-chain monounsaturated fatty acid that exerts the vial function in maintaining brain development and improving neural cells. Initial studies have shown that the self-repair of injured shark brains in a short period of time may be attributed to the repair and regeneration effect of NA on neural fibers in brain tissues [24]. In recent years, the regulatory functions of NA in neurological diseases have gradually been confirmed. It is reported that NA markedly improved motor skills, learning and memory abilities of mice with Alzheimer's disease [16]. In PD, NA was proven to improve liver inflammation in mice via modulating the inflammation and metabolism pathway. Additionally, the increase in neuroinflammation induced by MPTP in PD mice can be greatly restrained by NA therapy [17]. This study proved that NA treatment abolished the motor dysfunction caused by MPTP and restored the motor function of PD mice.

The loss of dopaminergic neurons and the α-syn agglomeration are the major pathological characteristics of PD [6]. Dopamine functions as the vital brain neurotransmitter released by dopaminergic neurons, the loss of which can result in deterioration of motor function. The α-syn aggregates can cause various cell dysfunction, activate neuroinflammation, and promote progressive neuronal death. TH is a rate-limiting enzyme in dopamine biosynthesis and is utilized by neurons to conflate dopamine after tyrosine intake [25]. Our study confirmed the elevation in protein and mRNA levels of α-syn as well as the decrease in protein and mRNA levels of TH in the MPTP-induced mouse model. In addition, dopamine levels were also inhibited under the induction of MPTP. However, NA administration notably reversed these phenomena to alleviate MPTP-induced neuronal injury. Additionally, in MPP-induced SH-SY5Y cells, we found that the low cell viability and high cell apoptosis rate induced by MPP were reversed by NA treatment.

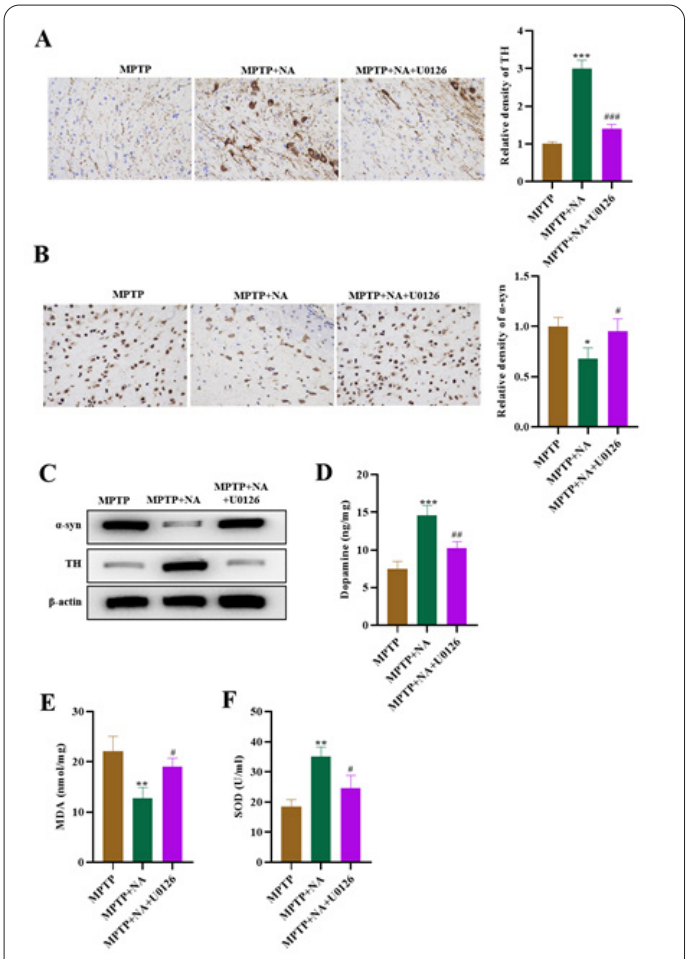

Fig. 4. The inhibitor of MEK/ERK pathway suppresses the therapeutic effect of NA on PD process. (A-B) IHC analysis of the density of TH or α-syn in SNc of mice in the MPTP group, MPTP+NA group, and MPTP+U0126 group. (C) Western blot outcomes of TH and α-syn levels in different groups. (D) Dopamine levels were tested in different groups. (E-F) The concentrations of MDA and SOD were tested via the commercial reagent kits. $^{*}p<0.05$, $^{\#}p<0.05$.

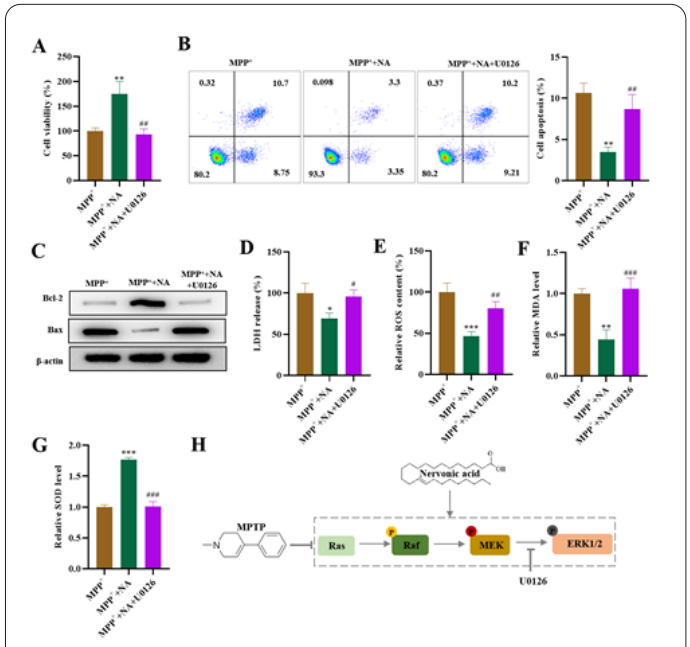

Fig. 5. NA inhibits neuronal apoptosis and oxidative stress through the MEK/ERK pathway. (A) CCK8 assay was performed to detect the viability of SH-SY5Y cells in the MPP group, MPP+NA group, and MPP+NA+U0126 group. (B) Cell apoptotic ratio was assessed through flow cytometry in different groups. (C) Western blot outcomes of Bcl-2 and Bax levels in different groups. (D) The production of LDH was tested in different cells. (E-G) The contents of ROS, MDA, and SOD were measured. $^{*}p<0.05$, $^{\#}p<0.05$.

Oxidative stress is an important cause of dopaminergic neuron apoptosis in PD. It is caused by ROS overproduction within cells, which attacks biological molecules such as DNA, proteins, and lipids, thereby affecting cell function. Studies have shown that mitochondrial dysfunction and ROS accumulation are two events associated with dopaminergic neuronal apoptosis [26, 27]. ROS overproduction causes damage to mitochondria and initiates degenerative processes, including activation of cell apoptosis and necrosis cascade reactions. It is reported that PD patients have elevated levels of lipid peroxidation and decreased activity of antioxidant enzymes [28]. Herein, we demonstrated that NA could reverse the oxidative stress characteristics induced by MPTP or MPP, including increased levels of ROS and MDA, as well as decreased levels of antioxidant enzyme SOD. Studies have confirmed that NA may function as an antioxidant mediator in human brains [29]. The previous study revealed that NA could protect fibroblasts in adrenoleukodystrophy from oxidative stress

damage [30]. NA can also enhance the production of intracellular ATP to avoid cell apoptosis and prevent mitochondrial damage [30]. In PD, we proved the antioxidant properties of NA, protecting neurons from oxidative damage and apoptosis.

MEK/ERK pathway is a highly conserved signaling cascade that transmits signals from cell surface receptors to facilitate cell proliferation [31]. Studies have confirmed that MEK/ERK pathway mainly stimulates cell growth and suppresses cell apoptosis [32]. ERK is a serine/threonine kinase and it has been proven to increase the survival rate of dopaminergic neurons [33]. The phosphorylation of ERK can be restrained by MPP stimulation in SH-SY5Y cells [33]. Chen et al. have suggested that icariin exerts a neuroprotective function in MPTP-induced PD mice through MEK/ERK pathway [34]. Additionally, it is proved that ERK inhibitors can enhance apoptosis and ROS production in H_2O_2-induced SH-SY5Y cells [35]. Herein, we proved that the induction of MPTP or MPP notably restrained the activity of MEK/ERK pathway by reducing the phosphorylation of MEK and ERK proteins. However, NA treatment restored their phosphorylation levels. Furthermore, we discovered that U0126 (pathway inhibitor) markedly reversed the neuroprotective and antioxidant effects of NA on MPTP mice and MPP^+ cells.

Taken together, this study proves that NA can reduce MPTP-induced neuronal damage and oxidative stress damage in PD mouse model and cell model via MEK/ERK Pathway. Our findings provide evidence for NA as a promising therapeutic drug for PD.

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