

# Aspirin attenuates L-DOPA-induced dyskinesia in hemi-parkinsonian rats

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## Research

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# Abstract

**Background:** At present, L-DOPA remained the gold standard therapy for motor symptoms of Parkinson's disease (PD) patients. However, prolonged administration of L-DOPA led to the development of dyskinesia. Aspirin, a non-steroidal anti-inflammatory drug (NSAID), had been widely used to relieve pain and inflammation. Recent studies indicated aspirin produced neuroprotection against dopamine (DA) neuronal loss in animal model. This study aimed to explore the effects of aspirin pre-treatment and co-treatment with L-DOPA on DA neurotoxicity and L-DOPA-induced dyskinesia (LID) as well.

**Methods:** Rats received a single 6-hydroxydopamine (6-OHDA) injection into substantia nigra to induce DA neuronal loss. For aspirin pre-treatment before L-DOPA studies: One day after 6-OHDA stimulation, rats were daily administered with aspirin for 3 weeks followed by daily L-DOPA treatment along with aspirin for additional 3 weeks. For aspirin co-treatment with L-DOPA studies: Three weeks after 6-OHDA administration, rats were daily treated by L-DOPA together with aspirin for another 3 weeks. DA neurotoxicity was analyzed via rat PD-like behavior test and DA neuronal counting. The movement disorders of dyskinesia triggered by L-DOPA were determined by the abnormal involuntary movements (AIM) scores analysis.

**Results:** we demonstrated both aspirin pre-treatment and co-treatment exerted anti-LID effects during L-DOPA treatment on 6-OHDA-lesioned rats. In addition, aspirin not only ameliorated DA neuronal damage, but also reduced the development of dyskinesia without affecting L-DOPA efficacy. Furtherly, inhibition of glial cells activation and the subsequent neuroinflammatory response might be involved in aspirin-attenuated dyskinesia.

**Conclusion:** the present study suggested aspirin could have beneficial potential to attenuate LID in PD.

## Background

Parkinson's disease (PD) is a chronic neurodegenerative disease, which affects 1% of the population over 60 year-old [1]. It is characterized by loss of nigrostriatal dopamine (DA) neurons in the substantia nigra (SN) of the brain [2]. The symptoms of motor dysfunction in PD include tremors at rest, muscular rigidity, bradykinesia and postural instability.

Treatment with the DA precursor, L-3, 4-dihydroxyphenylalanine (L-DOPA), is the most effective symptomatic therapeutic choice for PD patients [3]. However, prolonged administration of L-DOPA leads to the development of abnormal involuntary movements (AIM), known as L-DOPA-induced dyskinesia (LID) [4]. Dyskinesia is unfavorable for the quality of life, sometimes being more disabling than PD itself. Once dyskinesia appears, L-DOPA had little effects on PD symptoms, therefore remaining a significant challenge to mitigate several non-motor and motor impairment [5]. Therefore, strategies of promising synergistic effects in combination with L-DOPA might present a novel adjuvant therapy for PD.

Aspirin known as Acetylsalicylic Acid (ASA), a non-steroidal anti-inflammatory drug (NSAID), is widely used in 20th century to relieve pain and inflammation [6]. Recently, this oldest agent in medicine has been considered to be a potential new therapy for a range of neuropsychiatric disorders. A battery of studies confirmed that ASA had beneficial effects on mood disorders and schizophrenia. Also, ASA is associated with a reduced risk of Alzheimer's disease (AD) and PD [7, 8]. In addition, ASA produced neuroprotection against 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)-induced DA neuronal loss [9]. This information might open the prospect of ASA for neurological disorders treatment.

The current study aimed to investigate the effects of ASA pre-treatment and co-treatment with L-DOPA on DA neurotoxicity and L-DOPA-induced dyskinesia as well. Rats lesioned with 6-hydroxydopamine (6-OHDA) in SN were employed and treated with L-DOPA and ASA. DA neurotoxicity was analyzed via rat PD-like behavior test and DA neuronal counting. The movement disorders elicited by L-DOPA were determined by the AIM scores analysis. Especially, these findings might provide a more potential synergistic therapeutic strategy for PD.

## Methods

### Reagents

ASA and 6-OHDA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-tyrosine hydroxylase (TH, Catalog No. ab41528), cyclooxygenase 2 (COX-2, Catalog No.ab15191) antibodies were purchased from Abcam (Cambridge, MA, USA). Anti-ionized calcium-binding adapter molecule-1 (IBA-1, Catalog No.10904-1-AP), glia fibrillary acidic protein (GFAP, Catalog No. 16825-1-AP), interleukin-6 (IL-6, Catalog No. 21865-1-AP), inducible nitric oxide synthase (iNOS, Catalog No.18985-1-AP), $\beta$ -actin (Catalog No. 20536-1-AP) and Rabbit IgG (Catalog No. SA00001-2) antibodies were purchased from Proteintech Group (Chicago, IL, USA).

### Animals

Male Sprague-Dawley rats (200–250 g) were obtained from the Experimental Animal Center of the Third Military Medical University (Chongqing, China; Specific pathogen-free Grade II; Certificate No. SCXK 2012-0005). All rats were maintained under specific pathogen-free conditions. All animal experiments were performed in accordance with National Institute of Health Guideline for the Animal Care and Use of Laboratory Animal and the protocols were approved by the institutional Animal Care and Use Committee at Zunyi Medical University (Zunyi, China).

### 6-OHDA Lesions and ASA and L-DOPA Treatments

Rats received a single 6-OHDA (8  $\mu$ g in 4  $\mu$ l saline, Sigma-Aldrich (St Louis, MO, USA) unilateral injection into the SN pars compacta (coordinates ML + 2.2 mm, AP + 5.2 mm, DV -8.0 mm) on the right side of brain. For ASA pre-treatment before L-DOPA studies: one day after 6-OHDA stimulation, rats were daily administrated with ASA (10 mg/kg, p.o.) for 3 weeks followed by daily treatment of L-DOPA (25 mg/kg, i.p.) along with ASA (10 mg/kg, p.o.) for additional 3 weeks [10]. For ASA co-treatment with L-DOPA

studies: three weeks after 6-OHDA administration, rats were daily treated by L-DOPA (25 mg/kg, i.p.) together with ASA (10 mg/kg, p.o.) for another 3 weeks.

## Rotarod Test

Rotarod test was performed to study the muscular coordination. Rats were permitted to remain stationary for a while at 0 rpm. Then, the increasing speed from 10 rpm to 30 rpm over a 300-s period until animals fell off from rungs [11]. Rat behavior changes were detected and the mean duration time stayed on rod was recorded.

## AIM Scores Analysis

To assess LID-like behavioral manifestations, AIM scores were employed to assess the properties of L-DOPA and ASA on rat behavior changes at 3 time points (7, 14 and 21 days after 6-OHDA treatment). This scoring system was considered to be comparable to reflect human dyskinetic behavior [12, 13]. Rat dyskinetic behaviors were quantified based on frequency during a 1 min- monitoring period within 4 different 30-min blocks for a total of 120 min. Next, the total AIM score corresponded to the sum of the individual scores for 3 AIM subtypes (axial, limb and orolingual) were summed [14]. For each subtype, the dyskinesia severity was scored by a 4-point scale (0: absent; 1: present less than 50% of the time; 2: present more than 50% of the time; 3: continuous but interrupted by strong sensory stimuli; and 4: continuous, with no interruption by strong sensory stimuli).

## Forepaw Adjusting Steps (FAS) Test

The FAS test was applied to detect akinesia, a cardinal symptom of PD. Rats with unilateral DA depletion performed poorly stepping ability on the FAS test with the lesioned side of the body [15]. L-DOPA reversed this stepping deficit and thus FAS test could be used to determine whether any adjunctive treatment affected the efficacy of L-DOPA-mediated anti-parkinsonian [16]. For this test, experimenters were blinded to treatment condition, both hind legs and one forepaw were held, such that rat was bearing its weight on the forepaw to be tested. Rats were moved through the table at a speed of 90 cm/10 s and the number of adjusting steps taken on the weight-bearing forepaw was recorded. Rats were dragged for 6 trials per forelimb: 3 backhand and 3 forehand trials. Data were represented as the sum of the 3 trials per direction for each forepaw. The stepping results were shown as % intact stepping (lesioned steps/ intact steps). The lower % intact stepping score indicated the greater forelimb akinesia [17].

## Immunohistochemistry Staining

DA neurons were identified with anti-TH antibody, while microglia and astroglia were recognized with anti-IBA-1 and GFAP antibodies, respectively. Rats were sacrificed and perfused with PBS and 4% paraformaldehyde. Then, brains were fixed with 4% paraformaldehyde, and dehydrated with 30% sucrose solution until the brain sunk to the bottom at 4 °C. Rat brains were cut into 35 µm-transverse free-floating sections by freezing microtome. The section was stained with primary antibodies targeting TH antibody (1:800), IBA-1(1:1000) and GFAP (1:1000), respectively, followed by treatment with secondary antibodies. Images were acquired through an Olympus microscope with an attached Polaroid digital microscope

camera (Polaroid®, Cambridge, MA, USA). Quantification of TH-positive neuronal cell bodies was performed blindly by two investigators. Then, the mean value for SN TH-positive neuronal numbers was deduced by averaging the counts of 6 sections for each brain.

## Western Blot Analysis

Brain tissues were homogenated in cold PBS and lysed in a radio immunoprecipitation (RIPA) lysis buffer. Subsequently, the lysate was centrifuged at  $12,000 \times g$  for 15 min at  $4^{\circ}\text{C}$ . The protein concentrations were quantified using bicinchoninic acid (BCA) protein assay. The protein samples (10  $\mu\text{g}$ ) were separated by SDS-polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene fluoride (PVDF) membrane [18]. Then, the membrane were blocked via 5% non-fat Milk and incubated with the following primary antibodies:  $\beta$ -actin (1: 4000), TH (1: 1000), GFAP (1: 800), IBA-1 (1: 1000), COX-2 (1: 1200), IL-6 (1: 600), iNOS (1: 1000) and horseradish peroxidase-conjugated secondary antibodies (1: 4000). The blot was visualized by the enhanced ECL reagent.

## Statistical Analysis

Data were indicated as mean  $\pm$  standard error of the mean (SEM). Statistical significance was analyzed by one- or two-way ANOVA through the GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). When ANOVA demonstrated the significant differences, pairwise comparison between means was evaluated by Bonferroni's post hoc t test with correction. A value of  $p < 0.05$  was considered statistically significant.

## Results

### ASA Exerted Anti-Dyskinetic Effects within L-DOPA Treatment on 6-OHDA-Lesioned Rats

Rat LID behavior changes were evaluated by AIM scores. As shown in Fig. 1, L-DOPA aggravated the behavior changes of axial (Fig. 1B), limb (Fig. 1C), orolingual (Fig. 1D) and total AIM scores (Fig. 1E). However, pre-treatment of ASA attenuated L-DOPA-induced AIM scores aggravation. Similarly, co-treatment of ASA with L-DOPA reduced L-DOPA-increased these total and subtypes AIM scores (Fig. 2). Collectively, these results suggested that both ASA pre-treatment and co-treatment with L-DOPA suppressed the development of dyskinesia during L-DOPA treated 6-OHDA-lesioned rats.

### ASA Ameliorated 6-OHDA-Induced DA Neuronal Damage

The effects of ASA pre-treatment and co-treatment with L-DOPA on 6-OHDA-induced DA neuronal loss were investigated. For ASA pre-treatment before L-DOPA, as shown in Fig. 3A, ASA produced DA neuroprotection against 6-OHDA-caused decrease of DA neuronal number from 35 days after ASA treatment. However, L-DOPA couldn't prevent 6-OHDA-elicited DA neuronal damage. Interestingly, ASA

combined with L-DOPA conferred neuroprotection from 28 days after ASA administration. Consistent with DA neuronal counting analysis, similar results were indicated in TH protein detection (Fig. 3B).

For ASA co-treatment with L-DOPA, as shown in Fig. 4A and C, ASA protected against 6-OHDA-induced DA neuronal loss after ASA treatment for 7 and 21 days (relative to 28 and 42 days after 6-OHDA injection), whereas L-DOPA presented no neuroprotection in 6-OHDA-lesioned DA neurons. However, no significant difference of DA neuroprotection between 6-OHDA + ASA and 6-OHDA + ASA + L-DOPA groups was indicated. Same results were exhibited in TH protein expression measurement (Fig. 4B and D).

## **ASA Treatment Didn't Affect L-DOPA-Generated Anti-Parkinsonian Efficacy**

First, 6-OHDA-induced behavior changes were analyzed via rotarod test (Fig. 5A). For ASA pre-treatment, L-DOPA attenuated 6-OHDA-induced decrease of the time rat stayed on rod from L-DOPA treatment for 7 days (relative to 28 days after 6-OHDA injection). ASA ameliorated rat behavior changes after ASA pre-treatment for 35 days (relative to 35 days after 6-OHDA injection). Also, ASA combined with L-DOPA improved 6-OHDA-induced rat behavior dysfunctions. For ASA co-treatment, both L-DOPA alone and L-DOPA combined with ASA treatment attenuated behavior changes, while no significant improvement in ASA alone treatment group was shown. Then, whether ASA treatment affected L-DOPA-generated anti-parkinsonian efficacy was determined. Rats were still treated with above anti-dyskinetic dose of ASA and the motor performance was assessed via the FAS test. As shown in Fig. 5B, L-DOPA reversed 6-OHDA-caused stepping deficits and both ASA pre-treatment and co-treatment with L-DOPA preserved L-DOPA-exerted anti-parkinsonian efficacy.

## **ASA Modulated Glial Cells Activation during L-DOPA-Mediated Anti-Parkinsonian Effects**

The effects of ASA on glial cells activation in L-DOPA treatment against 6-OHDA-elicited DA neuronal damage were analyzed by immunofluorescence staining and western blot assay. As shown in Fig. 6, seven days after L-DOPA and ASA co-treatment (relative to 28 days after 6-OHDA administration), compared with the control group, 6-OHDA induced microglia and astroglia activation evidenced by the increased number of IBA-1-positive microglia and GFAP-positive astroglia and protein expressions of IBA-1 and GFAP. However, ASA alone, L-DOPA alone and ASA co-treatment with L-DOPA had no significant effects on glial cells activation. Twenty-one days after treatment (relative to 42 days after 6-OHDA application), ASA and ASA + L-DOPA attenuated 6-OHDA-induced astroglia activation, whereas no significant effects were shown after L-DOPA alone treatment. Moreover, ASA combined with L-DOPA inhibited IBA-1 protein expression although ASA or L-DOPA alone had no obvious inhibitory effects on microglial activation.

Next, pro-inflammatory mediators protein expressions in rat midbrain were detected. As shown in Fig. 7, compared with the control group, the protein expressions of COX-2, IL-6 and iNOS in 6-OHDA group were

increased 28 and 42 days after 6-OHDA stimulation. ASA co-treatment with L-DOPA reduced 6-OHDA-induced increase of these inflammatory mediators protein expressions 42 days after 6-OHDA application, whereas ASA or L-DOPA alone treatment had no significant actions.

## Discussion

The present study demonstrated both aspirin pre-treatment and co-treatment exerted anti-LID effects during L-DOPA treatment on 6-OHDA-lesioned rats. In addition, aspirin not only ameliorated DA neuronal damage, but also reduced the development of dyskinesia without affecting L-DOPA efficacy. Furtherly, inhibition of glial cells activation and the subsequent neuroinflammatory response might be involved in aspirin-attenuated dyskinesia.

L-DOPA has long been the standard therapy for treating symptoms of PD via the supplement of exogenous dopamine, which reverses the distinctive behavioral manifestations of PD. However, the eventual onset of motor fluctuations and LID complicates its utility in advanced PD [19–21]. In recent decades, various therapies have been developed for the treatment of LID. Nevertheless, PD patients remain disabled by the effects of LID. Amantadine is the only compound with anti-dyskinetic effects in PD patients, but several lines of evidence have demonstrated Amantadine produced short duration anti-dyskinetic effects [22]. Therefore, unveiling strategies to ameliorate LID still maintains a critical clinical hurdle [23]. ASA has been verified to exert neuroprotective effects in MPTP- and 6-OHDA-induced animal model against DA neuronal loss [24–26]. Therefore, ASA-mediated neuroprotection attracts an increasing attention. In this study, pre-treatment and co-treatment of ASA with L-DOPA attenuated L-DOPA-triggered dyskinesia in 6-OHDA-lesioned rats. Furthermore, the present study showed that L-DOPA attenuated motor symptoms of PD animal model rather than protecting DA neurons against 6-OHDA-induced neurotoxicity. These findings confirmed the critical limitations of L-DOPA since its effects were merely symptomatic and not as fundamental. In addition, one such limitation concerned that L-DOPA might accelerate DA neuronal loss during the progression of PD [27]. Moreover, pre-treatment and co-treatment of ASA with L-DOPA still generated DA neuroprotection and ameliorated LID without affecting the therapeutic actions of L-DOPA, suggesting that ASA might be repurposed as an adjunct treatment to mitigate LID in PD therapy.

The mechanisms underlying ASA-mediated neuroprotection were controversial, but appeared to include the inhibition of neuroinflammation. Neuroinflammation has been considered to contribute not only to PD progression, but also to development of LID [28, 29]. Substantia nigra is rich in microglia and astroglia, which are the main actors in neuroinflammatory responses, and their double role at the interface between immune and neurophysiological responses was widely studied [30]. Previous studies demonstrated that ASA down-regulated the inflammatory condition in activated microglia induced by lipopolysaccharide (LPS) [31]. In the present study, co-administration of ASA and L-DOPA reduced astroglia and microglia activation and inhibited inflammatory response 21 days not 7 days after ASA and L-DOPA treatment. These data implied the ability of ASA-attenuated LID might be associated with the inhibition of neuroinflammation. Recently, the underlying mechanisms of LID have been focused on L-DOPA-induced

inflammatory responses [32]. Although pre-clinical studies supported the role of neuroinflammation on LID, whether an exacerbated neuroinflammation might participate in the development of LID in PD patients was not elucidated yet. Thus, the mechanisms how L-DOPA promoted the neuroinflammatory responses, and how that would in turn influence the dyskinesia outcomes, warrant further exploration.

At present, this study provides the first evidence for the synergistic therapeutic strategy of ASA combined with L-DOPA in PD and LID. Therefore, to develop ASA effective treatment to stop or delay the progression of PD and LID, additional studies are needed to prove the underlying mechanisms and confirmed anti-dyskinetic effects of ASA in other animal models and even PD patients with LID.

## Conclusions

This study demonstrated ASA protected from DA neuronal damage and attenuated LID without affecting L-DOPA efficacy. These findings suggest ASA might be a potential alternative for attenuating LID in PD.

## Abbreviations

6-OHDA:6-hydroxydopamine; AD:Alzheimer's disease; AIM:Abnormal involuntary movements; ASA:Acetylsalicylic Acid; COX-2:cyclooxygenase 2; DA:dopamine; FAS:Forepaw Adjusting Steps; GFAP:glia fibrillary acidic protein; IBA-1:ionized calcium-binding adapter molecule-1; IL-6:interleukin-6; iNOS:inducible nitric oxide synthase; L-DOPA:L-3, 4-dihydroxyphenylalanine; LID:L-DOPA-induced dyskinesia; NSAID:non-steroidal anti-inflammatory; PD:Parkinson's disease; SN:substantia nigra; TH:tyrosine hydroxylase

## Declarations

### Ethics approval and consent to participate

All animal experiments were performed in accordance with National Institute of Health Guideline for the Animal Care and Use of Laboratory Animal and the protocols were approved by the institutional Animal Care and Use Committee at Zunyi Medical University (Zunyi, China).

### Consent for publication

Not applicable.

### Availability of data and materials

All data mentioned in this article are available from the corresponding author on reasonable request.

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### **Authors' contributions**

FZ conceived and designed the experiments. GFZ and CQZ participated in the experiments performance and DDL, YZZ, JJJ, GQW and FZ finished data analysis. FZ wrote, revised and checked the article. All authors read and revised and approved the final manuscript.

### **Competing interests**

The authors have declared no any conflict of interests.

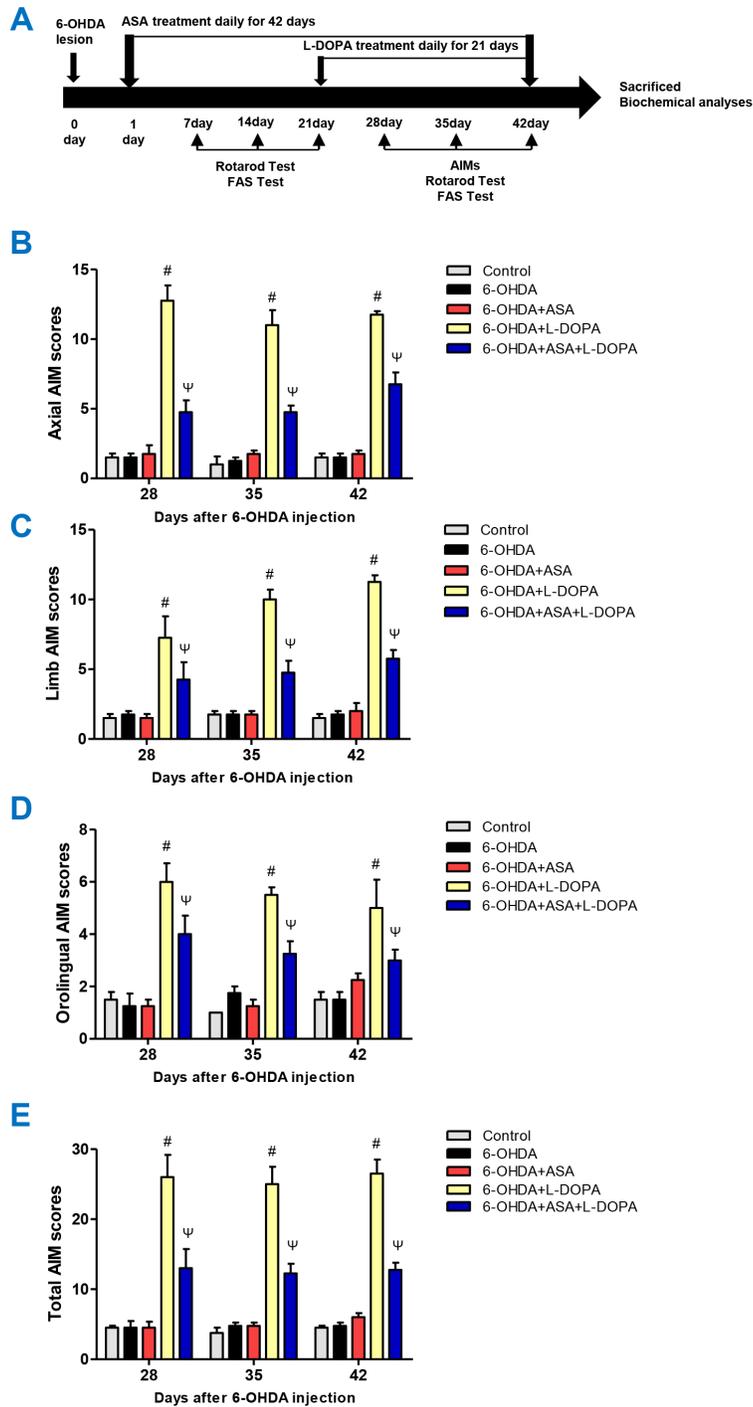
## **References**

- [1] Samii A, Al E. Parkinson's disease. *Lancet* 2004;363(9423):1783-93.
- [2] Kordower JH, C Warren O, Dodiya HB, Yaping C, Beach TG, Adler CH, Halliday GM, Bartus RT. Disease duration and the integrity of the nigrostriatal system in Parkinson's disease. *Brain* 2013;136(8):2419-31.
- [3] Antonini A. Levodopa in the treatment of Parkinson's disease: an old drug still going strong. *Clin Interv Aging* 2010;5:229-38.
- [4] Sgroi S, Kaelin-Lang A, Capper-Loup C. Spontaneous locomotor activity and L-DOPA-induced dyskinesia are not linked in 6-OHDA parkinsonian rats. *Front Behav Neurosci* 2014;8(4):331.
- [5] Encarnacion E, Hauser R. Levodopa-induced dyskinesias in Parkinson's disease: etiology, impact on quality of life, and treatments. *Eur Neurol* 2008;60(2):57-66.
- [6] Montinari MR, Minelli S, De Caterina R. The first 3500 years of aspirin history from its roots – A concise summary. *Vascul Pharmacol.* 2019;113:1-8.
- [7] Zhang C, Wang Y, Wang D, Zhang J, Zhang F. NSAID Exposure and Risk of Alzheimer's Disease: An Updated Meta-Analysis From Cohort Studies. *Front Aging Neurosci* 2018;10:83.
- [8] Ayyadevara S, Balasubramaniam M, Kakraba S, Alla R, Mehta JL, Shmookler Reis RJ. Aspirin-Mediated acetylation protects against multiple neurodegenerative pathologies by impeding protein aggregation. *Antioxid Redox Signal* 2017;27(17):1383-96.
- [9] Maharaj H, Maharaj DS, Daya S. Acetylsalicylic acid and acetaminophen protect against MPP<sup>+</sup>-induced mitochondrial damage and superoxide anion generation. *Life Sci* 2006;78(21):2438-43.

- [10] Jiao M, Hai Z, Lei-Ping Y, Pei-Hua S, Guo-Zhang J, Xuechu Z. L-stepholidine reduced L-DOPA-induced dyskinesia in 6-OHDA-lesioned rat model of Parkinson's disease. *Neurobiol Aging* 2010;31(6):926-36.
- [11] Khuwaja G, Khan MM, Ishrat T, Ahmad A, Raza SS, Ashafaq M, Javed H, Khan MB, Khan A, Vaibhav K, Safhi MM, Islam F. Neuroprotective effects of curcumin on 6-hydroxydopamine-induced Parkinsonism in rats: Behavioral, neurochemical and immunohistochemical studies. *Brain Res* 2011;1368(1):254-63.
- [12] Ostock CY, Hallmark J, Palumbo N, Bhide N, Conti M, George JA, Bishop C. Modulation of L-DOPA's antiparkinsonian and dyskinesic effects by  $\alpha$ 2-noradrenergic receptors within the locus coeruleus. *Neuropharmacology* 2015;95:215-25.
- [13] Lu DS, Chen C, Zheng YX, Li DD, Wang GQ, Liu J, Shi J, Zhang F. Combination treatment of icariin and L-DOPA against 6-OHDA-Lesioned dopamine neurotoxicity. *Front Mol Neurosci* 2018;11:155-.
- [14] Xie C, Wang WW, Zhang S, Yuan ML, Che JY, Gan J, Song L, Yuan WE, Liu ZG. Levodopa/benserazide microsphere (LBM) prevents L-dopa induced dyskinesia by inactivation of the DR1/PKA/P-tau pathway in 6-OHDA-lesioned Parkinson's rats. *Sci Rep* 2014;4:7506.
- [15] David L, Ostock CY, Jaunaraajs KL, Eskow, Dupre KB, Barnum CJ, Nirmal B, Bishop C. Behavioral and cellular modulation of L-DOPA-induced dyskinesia by beta-adrenoceptor blockade in the 6-hydroxydopamine-lesioned rat. *J Pharmacol Exp Ther* 2011;337(3):755-65.
- [16] Alam S. The partial 5-HT(1A) agonist buspirone reduces the expression and development of L-DOPA-induced dyskinesia in rats and improves L-DOPA efficacy. *Pharmacol Biochem Behav* 2007;87(3):306-14.
- [17] Chotibut T, Meadows S, Kasanga EA, McInnis T, Cantu MA, Bishop C, Salvatore MF. Ceftriaxone reduces L-dopa-induced dyskinesia severity in 6-hydroxydopamine parkinson's disease model. *Mov Disord* 2017;32(11):1547-56.
- [18] Greene JG, Dingledine R, Greenamyre JT. Neuron-selective changes in RNA transcripts related to energy metabolism in toxic models of parkinsonism in rodents. *Neurobiol Dis* 2010;38(3):476-81.
- [19] Ahlskog JE, Muenter MD. Frequency of levodopa-related dyskinesias and motor fluctuations as estimated from the cumulative literature. *Mov Disord* 2001;16(3):448–58.
- [20] Grandas F, Galiano ML, Tabernerero C. Risk factors for levodopa-induced dyskinesias in Parkinson's disease. *J Neurol* 1999;246(12):1127-33.
- [21] Stocchi F, Tagliati M, Olanow CW. Treatment of levodopa-induced motor complications. *Mov Disord* 2010;23(S3):S599-S612.
- [22] Perez-Lloret S, Rascol O. Efficacy and safety of amantadine for the treatment of L-DOPA-induced dyskinesia. *J Neural Transm (Vienna)* 2018;125(8):1237-50.

- [23] Pilleri M, Antonini A. Therapeutic strategies to prevent and manage dyskinesias in Parkinson's disease. *Expert Opin Drug Saf* 2014;14(2):281-94.
- [24] Aubin N, Curet O, Deffois A, Carter C. Aspirin and salicylate protect against MPTP-induced dopamine depletion in mice. *J Neurochem* 1998;71:1635-42.
- [25] Carrasco E, Werner P. Selective destruction of dopaminergic neurons by low concentrations of 6-OHDA and MPP<sup>+</sup>: protection by acetylsalicylic acid aspirin. *Parkinsonism Relat Disord* 2002;8(6):407-11.
- [26] Di Matteo V, Pierucci M, Di Giovanni G, Di Santo A, Poggi A, Benigno A, Esposito E. Aspirin protects striatal dopaminergic neurons from neurotoxin-induced degeneration: an in vivo microdialysis study. *Brain Res* 2006;1095(1):167-77.
- [27] Huh E, Choi JG, Sim Y, Oh MS. An integrative approach to treat parkinson's disease: ukgansan complements L-Dopa by ameliorating dopaminergic neuronal damage and L-Dopa-induced dyskinesia in mice. *Front Aging Neurosci* 2018;10:431.
- [28] Carta AR, Mulas G, Bortolanza M, Duarte T, Pillai E, Fisone G, Vozari RR, Del-Bel E. L-DOPA-induced dyskinesia and neuroinflammation: do microglia and astrocytes play a role? *Eur J Neurosci* 2016;45(1):73-91.
- [29] Aldakheel A, Kalia LV, Lang AE. Pathogenesis-targeted, disease-modifying therapies in parkinson disease. *Neurotherapeutics* 2014;11(1):6-23.
- [30] Speck AE, Schamne MG, Aguiar AS, Cunha RA, Rui DP. Treadmill exercise attenuates L-DOPA-induced dyskinesia and increases striatal levels of glial cell-derived neurotrophic factor (GDNF) in hemiparkinsonian mice. *Mol Neurobiol* 2018;56:2944-2951.
- [31] Li W-Y, Li F-M, Zhou Y-F, Wen Z-M, Ma J, Ya K, Qian ZM. Aspirin down regulates hepcidin by inhibiting NF- $\kappa$ B and IL6/JAK2/STAT3 pathways in BV-2 microglial cells treated with lipopolysaccharide. *Int J Mol Sci* 2016;17(12):1921.
- [32] Lindenbach D, Ostock CY, Eskow Jaunarajs KL, Dupre KB, Barnum CJ, Bhide N, Bishop C. Behavioral and cellular modulation of L-DOPA-induced dyskinesia by beta-adrenoceptor blockade in the 6-hydroxydopamine-lesioned rat. *J Pharmacol Exp Ther* 2011;337(3):755-65.

## Figures

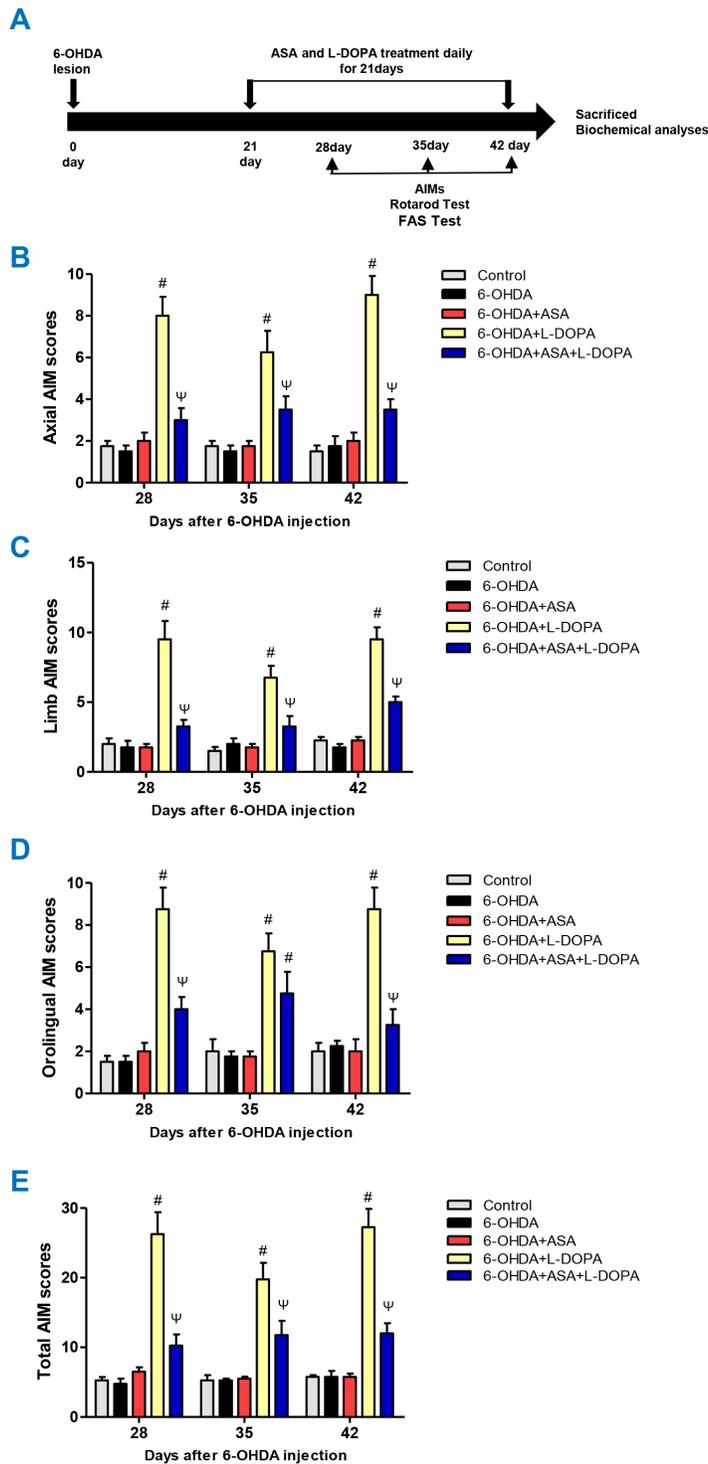


**Figure 1**

**Figure 1**

Pre-treatment of ASA exerted anti-dyskinetic effects within L-DOPA treatment on 6-OHDA-lesioned rats. Rats received a unilateral 6-OHDA (8  $\mu$ g) injection to SN pars compacta on right side of rat brain. One day later, rats were daily administrated with ASA (10 mg/kg, p.o.) for 3 weeks followed by daily treatment of L-DOPA (25 mg/kg, i.p.) for additional 3 weeks. Experimental design for 6-OHDA lesion, drug treatment and behavioral tests was shown (A). The effects of ASA on L-DOPA-induced behavior changes of axial

(B), limb (C), orolingual (D) and total AIM scores (E) were evaluated 28, 35 and 42 days after 6-OHDA administration, respectively. Data were mean  $\pm$  SEM from 6 rats. # $p$ <0.05 compared with 6-OHDA group;  $\Psi$  $p$ <0.05 compared with 6-OHDA+L-DOPA group.



**Figure 2**

**Figure 2**

Co-treatment of ASA with L-DOPA alleviated LID in 6-OHDA-lesioned rats. Rats were given a single unilateral injection of 6-OHDA (8  $\mu$ g) into rat SN pars compacta on right side of brain. Three weeks later,

rats were daily administrated L-DOPA (25 mg/kg, i.p.) along with ASA (10 mg/kg, p.o.) for another 3 weeks. Time schedule for drug treatment was indicated (A). After L-DOPA and ASA co-treatment, the effects of ASA on L-DOPA-elicited changes of axial (B), limb (C), orolingual (D) and total AIM scores (E) were assessed 28, 35 and 42 days after 6-OHDA application, respectively. Data were mean  $\pm$  SEM from 6 rats. # $p$ <0.05 compared with 6-OHDA group;  $\Psi$  $p$ <0.05 compared with 6-OHDA+L-DOPA group.

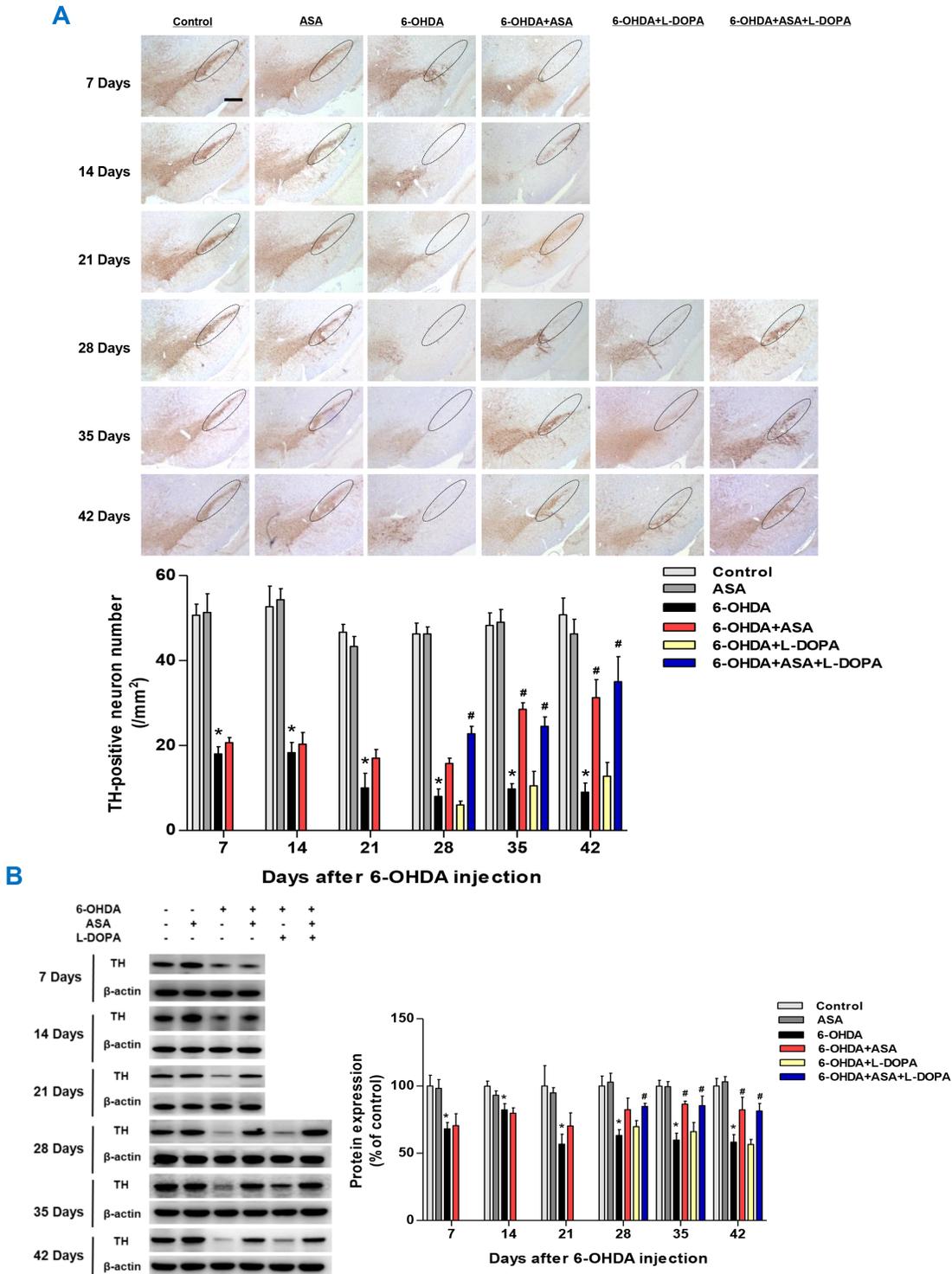
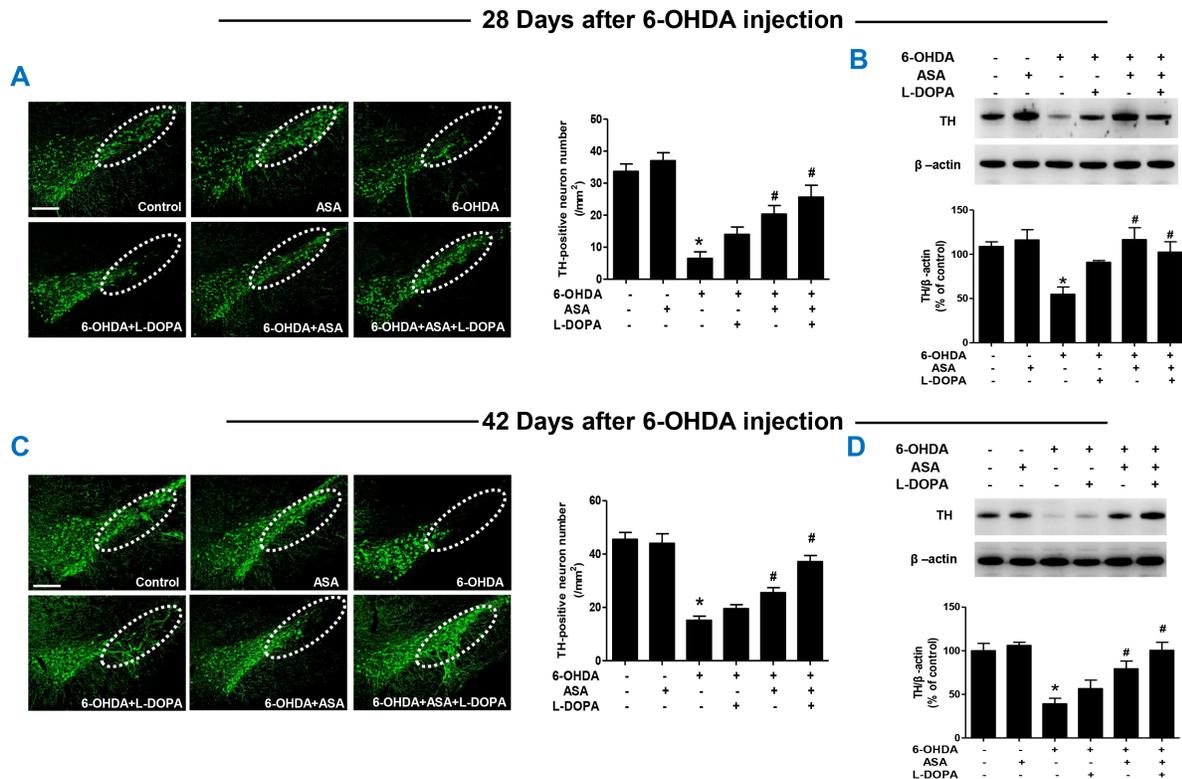


Figure 3

Figure 3

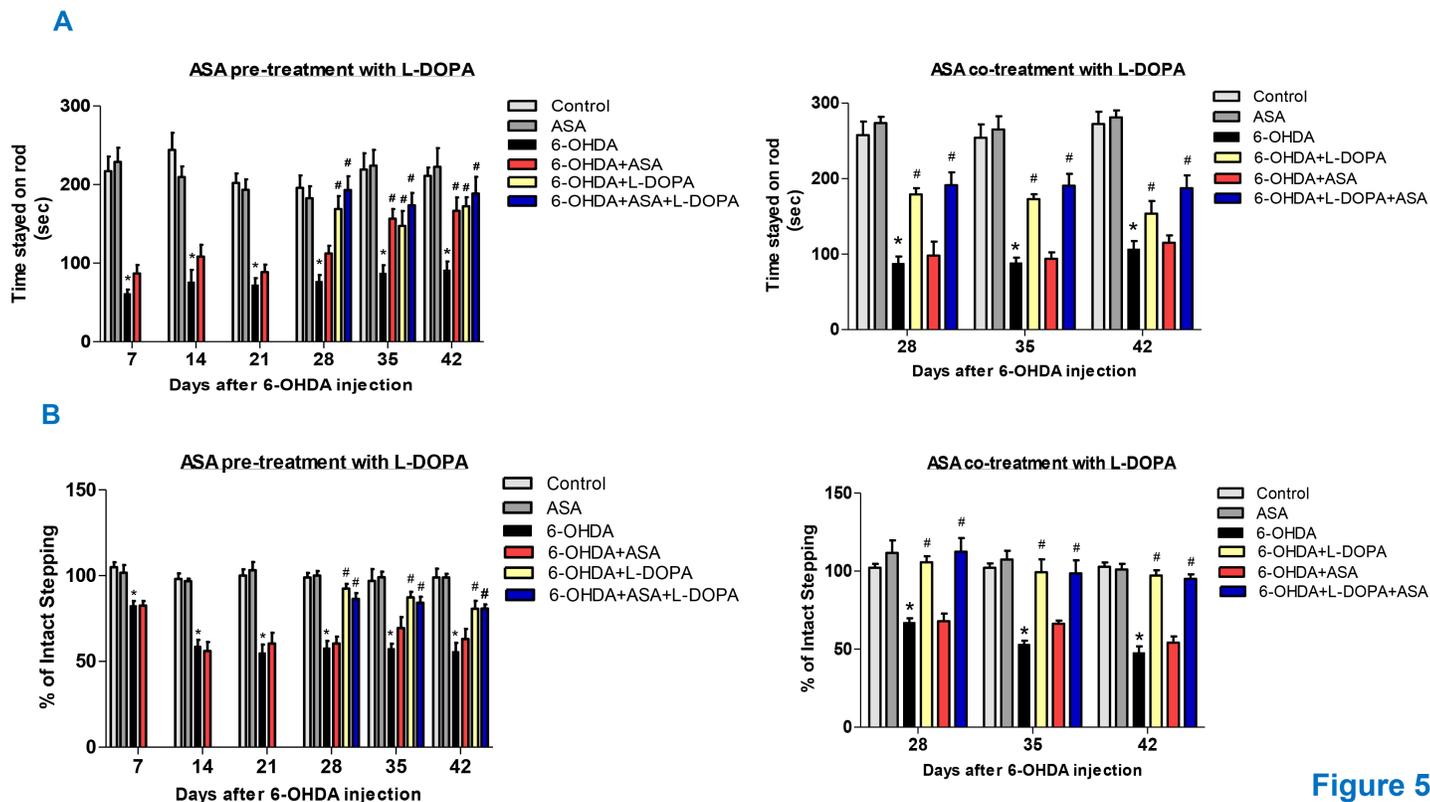
The effects of ASA pre-treatment before L-DOPA on 6-OHDA-induced DA neuronal loss. Seven, 14, 21, 28, 35 and 42 days after 6-OHDA injection, rats were sacrificed and brains were collected, respectively. Rat SN DA neurons in brain sections were immunostained and recognized with an anti-TH antibody and DA neuronal loss in the SN was analyzed via the quantification of TH-positive neurons (A). The “ellipse” presented the area of SN. Scale bar = 100  $\mu$ m. The protein level of TH was determined by western blotting. The ratio of densitometry values of TH with  $\beta$ -actin was assessed and normalized to each respective control group (B). Data were mean  $\pm$  SEM from 6 rats. \* $p$ <0.05 compared with the control group; # $p$ < 0.05 compared with 6-OHDA group.



**Figure 4**

**Figure 4**

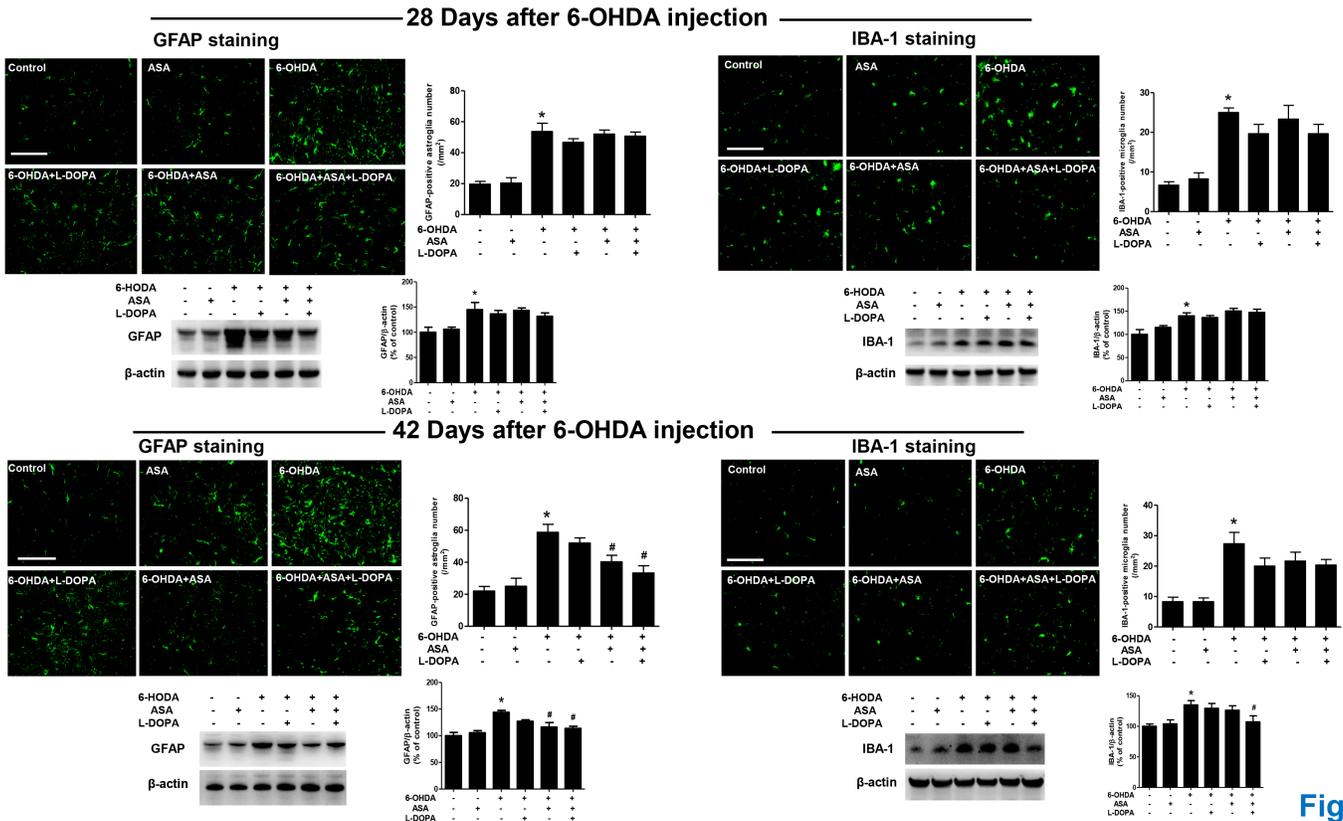
The effects of ASA co-treatment with L-DOPA on 6-OHDA-induced neurotoxicity. After L-DOPA and ASA co-treatment for 7 and 21 days (relative to 28 and 42 days after single 6-OHDA injection), respectively, DA neuronal lesion in SN was analyzed via the quantification of TH-positive neurons after immunostaining with an anti-TH antibody (A and C). The “ellipse” presented the area of SN. Scale bar = 100  $\mu$ m. TH protein level was determined by western blot assay. The ratio of densitometry values of TH with  $\beta$ -actin was assessed and normalized to each respective control group (B and D). Data were mean  $\pm$  SEM from 6 rats. \* $p$ <0.05 compared with the control group; # $p$ <0.05 compared with 6-OHDA group.



**Figure 5**

**Figure 5**

ASA treatment didn't affect L-DOPA-generated anti-parkinsonian efficacy. L-DOPA-generated beneficial effects on 6-OHDA-induced rat behavior changes were analyzed by rotarod test. The time stayed on the rod was recorded (A). The effects of L-DOPA combined with ASA on forelimb stepping changes were determined via the forepaw adjusting steps (FAS) test to further evaluate whether ASA affected L-DOPA efficacy (B). Data were mean  $\pm$  SEM from 6 rats. \* $p < 0.05$  compared with the control group; # $p < 0.05$  compared with 6-OHDA group.



**Figure 6**

**Figure 6**

ASA modulated glial cells activation during L-DOPA-mediated anti-parkinsonian effects. A single unilateral injection of 6-OHDA (8  $\mu$ g) into rat SN pars compacta on right side of brain was performed. Three weeks later, rats were daily given L-DOPA (25 mg/kg, i.p.) co-treatment with ASA (10 mg/kg, p.o.) for additional 3 weeks. Rat brains were sectioned and immunostained with anti-GFAP and IBA-1 antibodies 28 and 42 days after 6-OHDA stimulation. The images were representative of 6 rats in each group. Scale bar=100  $\mu$ m. The number analysis of SN IBA-1-positive microglia and GFAP-positive astroglia from 6 evenly spaced brain sections of each rat was performed. In addition, rat midbrains were collected to detect the protein expressions of GFAP and IBA-1 28 and 42 days after 6-OHDA treatment via western blotting. The ratio of densitometry values of GFAP and IBA-1 with  $\beta$ -actin was assessed and normalized to each respective control group. Data were mean  $\pm$  SEM from 6 rats. \* $p$ <0.05 compared with the control group; # $p$ < 0.05 compared with 6-OHDA group.

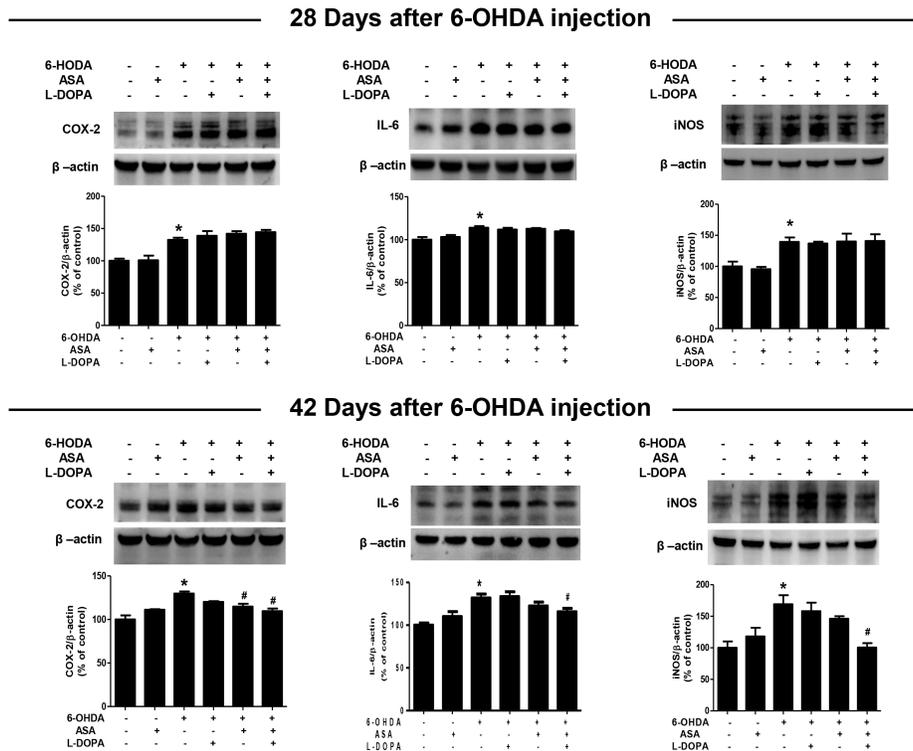


Figure 7

Figure 7

ASA inhibited pro-inflammatory mediators levels upon L-DOPA-generated anti-parkinsonian actions. Rats received a single injection of 6-OHDA (8 μg) into rat SN pars compacta on right side of brain. Three weeks later, rats were treated with L-DOPA (25 mg/kg, i.p.) along with ASA (10 mg/kg, p.o.) daily for another 3 weeks. The protein levels of rat midbrain pro-inflammatory mediators, such as COX-2, IL-6 and iNOS, were detected 28 and 42 days after 6-OHDA administration by western blot assay. Data were mean ± SEM from 6 rats. \*p<0.05 compared with the control group; #p< 0.05 compared with 6-OHDA group.