Hydroxysafflor yellow A promotes α-synuclein clearance via regulating autophagy in rotenone-induced Parkinson's disease mice

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Abstract

Autophagy is an intracellular degradation process that is involved in α-synuclein (α-syn) homeostasis and Parkinson's disease (PD). The purpose of this study was to investigate whether hydroxysafflor yellow A (HSYA) could promote α-syn clearance via regulating autophagy in PD mice. Male C57BL/6 mice were intraperitoneally treated with HSYA. Thirty minutes later, they were intragastrically administered with rotenone at a dose of 30 mg/kg. The hanging wire test was performed at 14 and 28 days. Then, autophagosomes and ultrastructural changes were examined by transmission electron microscopy. The expression of tyrosine hydroxylase (TH), α-syn, JNK1, p-JNK1, Bcl-2, p-Bcl-2, Beclin1, autophagy-related proteins (Atg) 7 and 12-5, and the LC3-II/LC3-I ratio were investigated by western blot. The hanging time of HSYA-treated PD mice was significantly increased compared with that of rotenone-induced PD mice (p < 0.05 or p < 0.01). Compared with rotenone-induced PD mice, treatment with HSYA augmented the formation of autophagosomes. The expression of TH, p-JNK1/JNK1, Beclin1, Atg7, Atg12-5, p-Bcl-2/Bcl-2, and the LC3-II/LC3-I ratio were significantly increased, whereas the expression of α-syn was reduced in the rotenone plus HSYA group. These results indicate that HSYA promoted α-syn clearance via regulating autophagy in rotenone-induced PD mice.

Key words: *hydroxysafflor yellow A, rotenone, neurotoxicity, α-synuclein, autophagy.*

Introduction

Over the past two decades, α-synuclein (α-syn) has been shown to play a crucial role in both familial and sporadic forms of Parkinson's disease (PD) [4]. First, point mutations in the *SNCA* gene that encodes the α-syn protein were demonstrated to cause PD with Lewy bodies [16, 17]. Then, it was shown that duplication or triplication of the α-syn locus was found in PD families and in sporadic cases [1]. Furthermore, patients with a triplication had an earlier onset, and more severe and faster progressing disease than those with duplication of the *SNCA* locus [2]. An important discovery was the identification of α-syn as a component of Lewy bodies in both familial and sporadic PD patients [20]. In spo-

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radic PD, α-syn accumulates in neuronal cell bodies and processes to form Lewy neurites in the brain, spinal cord, and peripheral nervous system [12]. To date, the exact mechanism by which α-syn accumulation causes neuronal loss and leads to the development of PD has not been elucidated. In fact, multiple organelles have been implicated in the toxicity of α-syn accumulation, including synaptic vesicles, mitochondria, and endoplasmic reticulum. Moreover, inter-organelle contact and organelle axonal transport are also disrupted by the toxicity of α-syn accumulation [24].

Autophagy is a catabolic process that mediates the degradation of proteins and dysfunctional organelles in eukaryotic cells. Autophagy can be divided into three main types: macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy plays a key role in the degradation of large portions of the cytoplasm, and even whole organelles, through the formation of double-membrane lipid structures called autophagosomes [10]. Autophagy is also involved in the degradation of aggregation-prone proteins, such as α-syn. Impairment of autophagy results in α-syn accumulation [15]. A growing body of evidence has demonstrated that there is a reduction of α-syn degradation in PD patients [3]. It was also reported that altered autophagy is associated with a variety of neuronal diseases characterised by neurodegeneration and movement disorders [19].

The characteristic symptoms of PD have been described in ancient Chinese medical books. Chinese herbal medicine has been used to treat PD patients for centuries. Herbal medicine is still popular for the treatment of PD in Asian countries, such as China, Japan, and Korea [22]. Recent reports have demonstrated that extracts isolated from Chinese herbal medicines can modulate autophagy and elicit an improvement in neurodegenerative diseases [14]. *Carthamus tinctorius* L, a Chinese herbal medicine, has been widely used for the treatment cerebrovascular disease. Hydroxysafflor yellow A (HSYA) is an active component of *Carthamus tinctorius* L. Previous findings from our laboratory showed that HSYA could alleviate the neurotoxicity of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine by inhibiting oxidative stress [8]. HSYA improved dopaminergic neuron integrity and motor function in a rodent model of PD by increasing the brain-derived neurotrophic factor (BDNF) level in the striatum [9]. The present study aimed to inves-

tigate whether HSYA promotes α-syn clearance via regulating autophagy in rotenone-induced PD mice.

Material and methods

Animals and HSYA

Eight-week-old male C57BL/6 mice were acquired from Jinan Pengyue Experimental Animal Co., Ltd. (Jinan, China). Animals were housed in a climate-controlled room, maintained on a 12-h/12-h light/dark cycle, and given food and water *ad libitum*. The experiments were performed according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (publication 86-23, revised in 1986) and approved by the Institutional Animal Ethics Committee of Yantai University. HSYA (98% purity by HPLC) was obtained from Shandong Luye Pharmaceutical Co., Ltd. (Yantai, China). HSYA is a hydrophilic compound and is stable in water. HYSA was dissolved in normal saline in the present experiment.

Experimental design

Mice were randomly divided into four groups (*n* = 12 per group): control, rotenone, HSYA, and HSYA + rotenone groups. Animals were intraperitoneally treated with either normal saline (control group and rotenone group) or 20 mg/kg HSYA (HSYA group and HSYA + rotenone group), once a day, for 28 days. Thirty minutes after each saline/HSYA administration, the rotenone and HSYA + rotenone groups were intragastrically administered with rotenone at a dose of 30 mg/kg for 28 days. The animals in the control and HSYA groups were given the same volume of 0.5% sodium carboxymethylcellulose.

Hanging wire test

The wire hanging apparatus comprised a stainless steel bar (50 cm length; 2 mm diameter) resting on two vertical supports and elevated 37 cm above a flat surface. This test was performed as previously described [18]. At 14 and 28 d of rotenone/HSYA treatment, mice were placed on the bar midway between the supports and were observed for 60 s in three trials. The amount of time spent hanging (hanging time) was recorded.

Transmission electron microscopy

The ultrastructure of autophagosomes from the striatum of animals was examined by transmission

electron microscopy. Briefly, striatal tissues (from $n = 3$ mice per group) were cut into 1-mm^3 sections, and fixed in 2.5% glutaraldehyde for 24 h, post-fixed with 1% osmic acid for one hour, dehydrated, and embedded. Tissues were cut into ultrathin sections of approximately 60 nm with a Leica EM UC7 ultra-microtome. The sections were stained with uranyl acetate and lead citrate. Then, the sections were observed using a Jeol JEM-1400 electron microscope.

Western blot

Striatal tissue was lysed in RIPA buffer and centrifuged at 12,000 g for 20 min at 4°C. Proteins (50 μg) were analysed using 8% or 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Then the proteins were transferred to polyvinylidene difluoride membranes at 110 V for 1 h. After blocking with 5% non-fat milk for 2 h, the membranes were incubated with the primary antibodies: rabbit anti-tyrosine hydroxylase (TH, 1 : 1000, Millipore), rabbit anti-α-syn (1 : 1000, Millipore), rabbit anti-p-JNK1 (1 : 1000; Cell Signaling Technology), rabbit anti-JNK1 (1 : 1000; Cell Signaling Technology), rabbit anti-Beclin1 (1 : 2000; Cell Signaling Technology), rabbit anti-p-Bcl-2 (1 : 2000; Cell Signaling Technology), rabbit anti-Bcl-2 (1 : 2000; Cell Signaling Technology), rabbit anti-Atg7 (1 : 1000; Cell Signaling Technology), rabbit anti-Atg12-5 (1 :

1000; Cell Signaling Technology), and mouse anti-LC3 (1 : 1000; Marine Biological Laboratory). After washing, the membranes were incubated with a horseradish peroxidase-labelled secondary antibody (mouse or rabbit, 1 : 2000; Beyotime Institute of Biotechnology). Bands were visualised using the ECL kit (Beyotime Institute of Biotechnology), and quantified using Image Quant LAS 4000 (GE Healthcare Bio-Sciences AB, Tokyo, Japan). Anti-β-actin (1 : 1000; Beyotime Institute of Biotechnology) served as the loading control.

Statistical analysis

Data were expressed as the mean ± SEM and analysed using SPSS 20.0 Statistical Software (IBM Corp., Armonk, NY, USA). Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's test. Statistical significance was defined as $p < 0.05$.

Results

Effect of HSYA on the hanging time in rotenone-induced PD mice

At 14 and 28 d after rotenone exposure, the hanging time of rotenone-induced PD mice was significantly decreased compared with the control group ($p < 0.01$). Compared with the rotenone group,
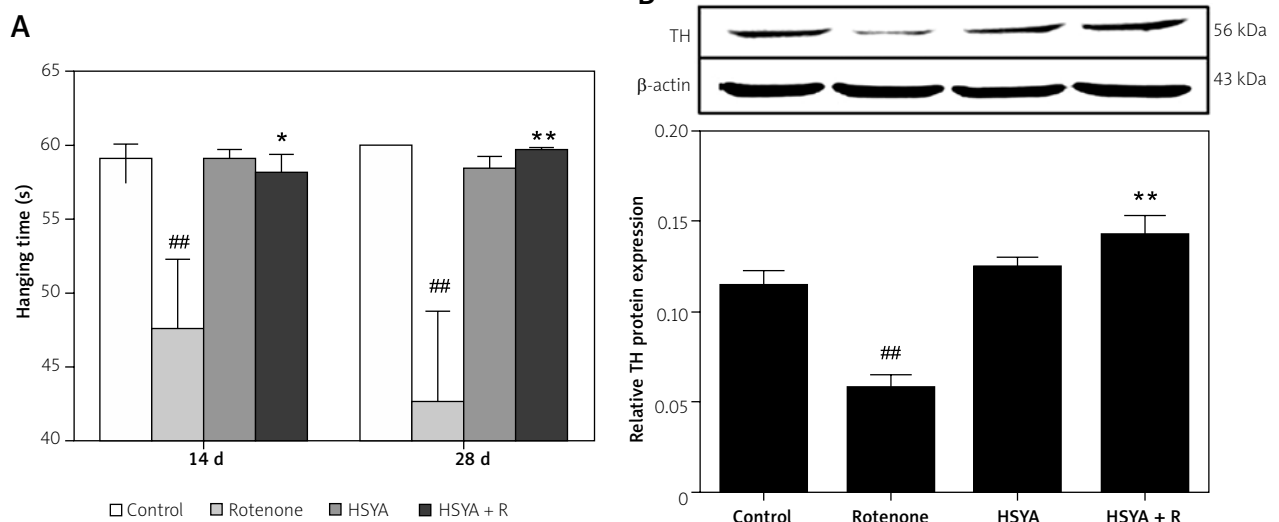

Fig. 1. Effect of hydroxysafflor yellow A (HSYA) on the hanging time ($n = 12$) and tyrosine hydroxylase (TH) content ($n = 6$) in rotenone-induced PD mice. Data are expressed as the mean ± SEM. Statistical analyses were performed using one-way analysis of variance (ANOVA) and Tukey's test; $^{##}p < 0.01$ compared with control group, $*p < 0.05$, $**p < 0.01$ compared with the rotenone group.

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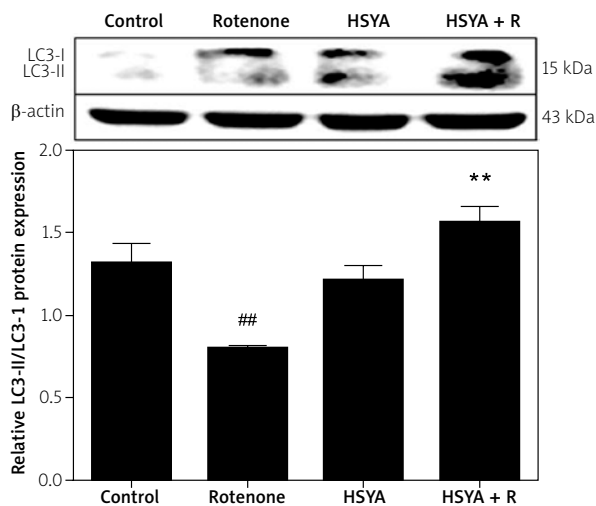

Fig. 2. Effect of hydroxysafflor yellow A (HSYA) on the LC3-II/LC3-I ratio in rotenone-induced PD mice. Data are expressed as the mean ± SEM ($n = 6$). Statistical analyses were performed using one-way analysis of variance (ANOVA) and Tukey's test; $^{\#\#}p < 0.01$ compared with the control group, $^{**}p < 0.01$ compared with the rotenone group.

the hanging time in the HSYA + rotenone group was significantly increased ($p < 0.05$ or $p < 0.01$). Compared with the control group, there was no significant change in the hanging time observed for the HSYA alone group (Fig. 1 A).

Effect of HSYA on the TH content in rotenone-induced PD mice

The TH content is shown in Fig. 1B. The TH content was decreased in the mouse striatal tissues 28 d after rotenone exposure. There was a significant increase in the TH content in the HSYA + rotenone group compared with the rotenone group ($p < 0.01$).

Effect of HSYA on autophagy in rotenone-induced PD mice

A significant decrease in the LC3-II/LC3-I ratio (autophagy marker) was observed in the rotenone-induced PD mice compared with the control group ($p < 0.01$). Compared with the rotenone group,
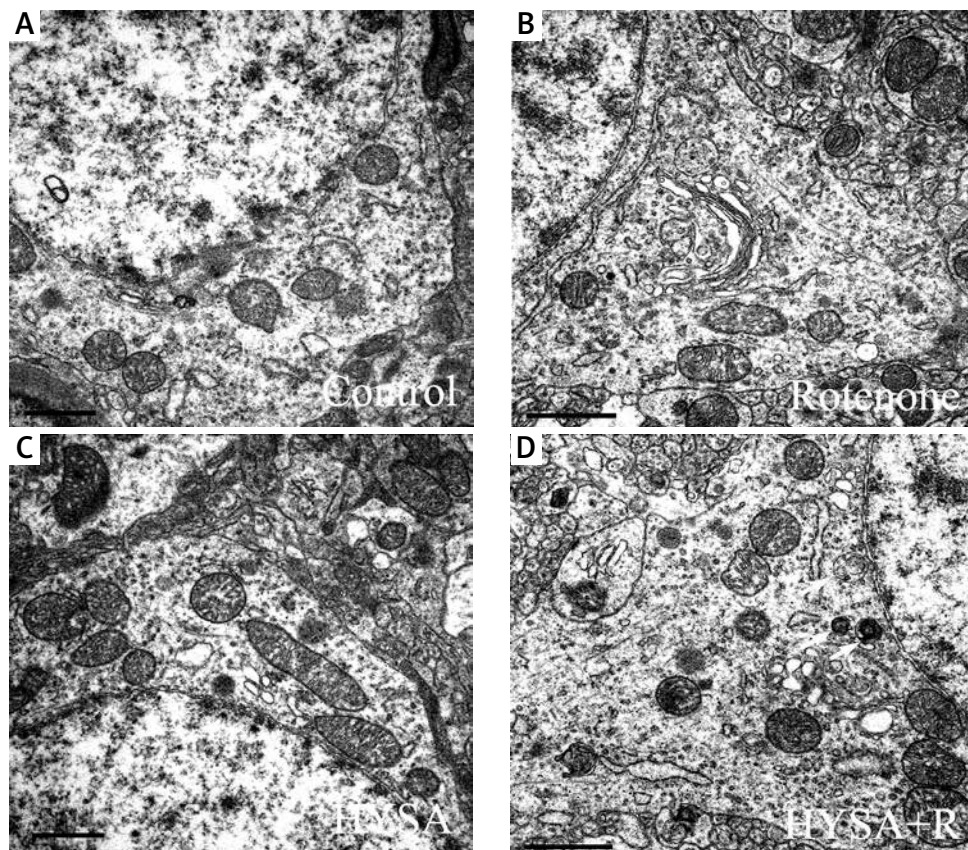

Fig. 3. Effect of hydroxysafflor yellow A (HSYA) on autophagosome formation in rotenone-induced PD mice. Typical double-membrane and lamellar autophagosomes were observed in tyrosine hydroxylase (TH) neurons of the HSYA + rotenone group. However, there were few autophagosomes observed in control, rotenone and HSYA groups ($n = 3$, Scale bar = 1 μm).

the HSYA + rotenone group showed a significant increase in the LC3-II/LC3-I ratio (Fig. 2, *p* < 0.01). The LC3-II/LC3-I ratio with HSYA treatment alone was the same as that of the control group (Fig. 2). The ultrastructure of autophagosomes was investigated by transmission electron microscopy (Fig. 3). There were typical double-membrane and lamellar autophagosomes in TH neurons of the HSYA + rotenone group, consistent with the LC3-II/LC3-I ratio.

Effect of HSYA on the α-syn content in rotenone-induced PD mice

The α-syn content in the rotenone group increased markedly compared with that of the control group (*p* < 0.01). However, treatment with HSYA attenuated the augmented α-syn content induced by rotenone (*p* < 0.01). Compared with the control group, there was no significant difference in the α-syn content of the HSYA alone group, indicating that the HSYA had no effect on the α-syn content in normal mice (Fig. 4).

Effect of HSYA on the levels of p-JNK1, p-Bcl-2, Beclin1, ATG7, and ATG12-5 in rotenone-induced PD mice

Using western blot analysis, we examined the effect of HSYA on the levels of p-JNK1, p-Bcl-2, Beclin1, ATG7, and ATG12-5 in rotenone-induced PD mice (Fig. 5). HSYA administration alone had no effect on the levels of these proteins when compared with their levels in mice of the control group. Compared with the control group, the levels of p-JNK1, p-Bcl-2, Beclin1, ATG7, and ATG12-5 in the rotenone group were significantly decreased (*p* < 0.01), whereas treatment with HSYA blocked their decrease (*p* < 0.05 or *p* < 0.01).

Discussion

The present data suggest that HSYA can promote α-syn clearance via regulating autophagy in a rotenone-induced PD model; α-syn is a protein implicated in PD and thought to be one of the main pathological drivers of the disease. Based on the present results, it is reasonable to speculate that the promotion of autophagy followed by an increase in α-syn clearance is one of the mechanisms underlying the neuroprotective effect of HSYA.

Various methods of evaluating motor impairment in PD models have been demonstrated, including catalepsy, locomotion reduction, and postural insta-

bility. The hanging wire test is a sensitive method for evaluating balance, coordination, and strength in PD animals [21]. In our study, impaired hanging wire performance was observed at 14 d and 28 d of rotenone exposure. TH is the rate-limiting enzyme in the synthesis of dopamine. A decrease in TH levels parallels the loss of dopaminergic neurons in PD patients [7]. The present findings showed that the TH levels in mice of the rotenone group decreased compared with the control group. Treatment with HSYA not only ameliorated the impaired hanging wire performance but also abated the reduction of TH level in the rotenone-induced PD model. These results suggested that HSYA can attenuate the injury to dopaminergic nerves and therefore improve the motor function of rotenone-induced PD mice.

Most assays for autophagy use LC3 as a substrate to measure autophagic flux. LC3 can be cleaved by Atg4 to form LC3-I. Then, LC3-I conjugates to phosphatidylethanolamine to form LC3-II. LC3-II is localised to isolation membranes and autophagosomes [11]. The LC3-II/LC3-I ratio is the most widely used autophagosome marker because it reflects the number of autophagosomes and autophagy-related
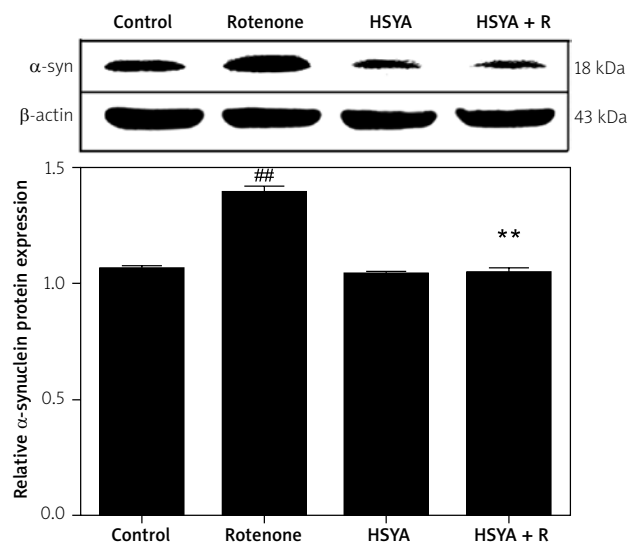

Fig. 4. Effect of hydroxysafflor yellow A (HSYA) on the α-synuclein content in rotenone-induced PD mice. Data are expressed as the mean ± SEM (*n* = 6). Statistical analyses were performed using one-way analysis of variance (ANOVA) and Tukey's test; ##*p* < 0.01 compared with the control group. **p* < 0.01 compared with the rotenone group.

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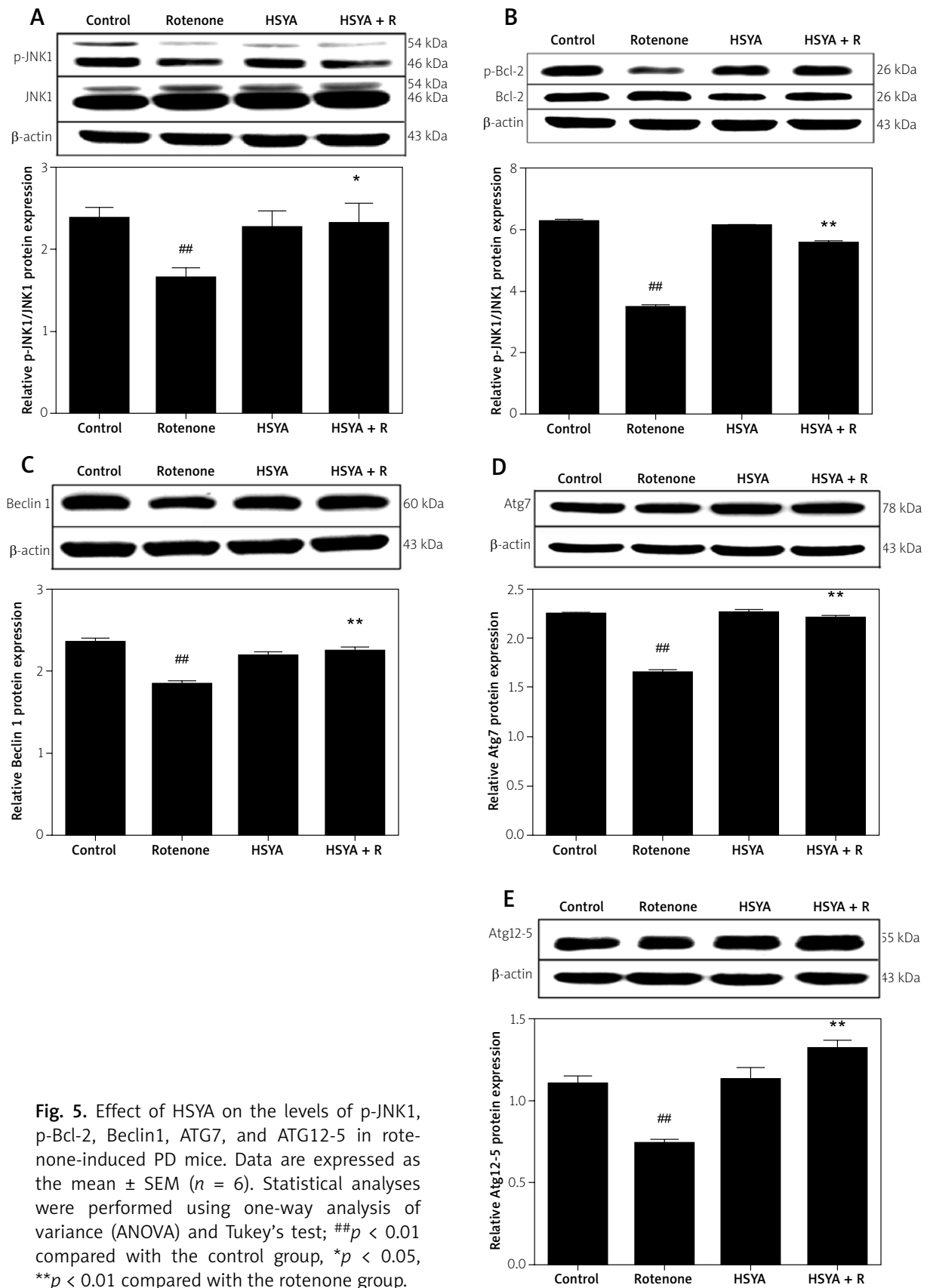

Fig. 5. Effect of HSYA on the levels of p-JNK1, p-Bcl-2, Beclin1, ATG7, and ATG12-5 in rotenone-induced PD mice. Data are expressed as the mean ± SEM (n = 6). Statistical analyses were performed using one-way analysis of variance (ANOVA) and Tukey's test; $^{\#\#}p < 0.01$ compared with the control group, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the rotenone group.

structures [6]. The increased LC3-II/LC3-I ratio and the existence of autophagosomes in the present study confirmed that HSYA enabled autophagosome formation in the rotenone-induced PD mice. The accumulation of α-syn promotes inclusion formation, which leads to the formation of Lewy bodies. One possible strategy to prevent PD is to decrease the soluble α-syn level. A previous study showed that autophagy plays a key role in α-syn degradation [5]. Accompanying the augmentation of autophagy, HSYA treatment reduced the α-syn level in rotenone-induced PD mice. These results indicate that HSYA can promote α-syn clearance via regulating autophagy.

The JNK1 signalling pathway has also been shown to regulate autophagy [13]. In mammalian cells, Bcl-2 binds to Beclin1 and inhibits the autophagy function of Beclin1. JNK1 activation increases Bcl-2 phosphorylation, then Bcl-2 dissociates from Beclin1 and stimulates autophagy by disrupting the Bcl-2/Beclin1 complex [23]. These findings are consistent with our observation that HSYA augmented the levels of p-JNK1 and p-Bcl-2, which would lead to the release of Beclin1 and an increase of autophagy-related proteins (ATG7, ATG12-5).

In summary, the present study suggested that HSYA can promote α-syn clearance via regulating autophagy in a PD model. The mechanism of pharmacological action of HSYA at least partly involved JNK1-mediated phosphorylation of Bcl-2, followed by disruption of the Bcl-2/Beclin1 complex (Fig. 6).

Acknowledgements

Disclosure

The authors report no conflict of interest.

Fig. 6. Schematic diagram of hydroxysafflor yellow A (HSYA) promoting α-synuclein clearance via regulating autophagy in rotenone-induced Parkinson's disease mice.

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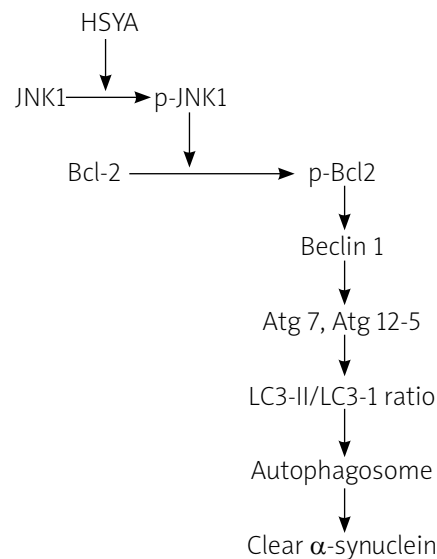2. Chartier-Harlin MC, Kachergus J, Roumier C, Mouroux V, Douay X, Lincoln S, Levecque C, Larvor L, Andrieux J, Hulihan M, Waucquier N, Defebvre L, Amouyel P, Farrer M, Destée A. Alpha-synuclein locus duplication as a cause of familial Parkinson's disease. Lancet 2004; 364: 1167-1169.
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