Neuroprotective effects of Kukoamine A on neurotoxin-induced Parkinson’s model through apoptosis inhibition and autophagy enhancement

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Article info

Article history:
Received 4 November 2016
Received in revised form 18 February 2017
Accepted 21 February 2017
Available online 24 February 2017

Keywords:
Parkinson’s disease (PD)
Kukoamine A (KuA)
Autophagy
Neurotoxin
SH-SY5Y cells
C57BL/6 mice

A B S T R A C T

Parkinson’s disease (PD) is characterized by the loss of dopaminergic neurons in substantia nigra (SN). Our previous study demonstrated Kukoamine A to exhibit strong neuroprotective effects through antioxidative stress, anti-inflammation, anti-excitotoxicity. In the present study, MPP⁺ and MPTP-induced PD models of cell and animal were used to investigate the effects of KuA on PD. Our results demonstrated that KuA ameliorated cell loss and mitochondrial membrane potential (MMP) loss, and inhibited Bax/Bcl-2 ratio and MAPKs family that were induced by MPP⁺. In addition, animal experiments showed that KuA improved the motor function and neuronal activity, and increased the positive cells of tyrosine hydroxylase (TH) both in substantia nigra (SN) and striatum (Str). Moreover, KuA could decrease the expression of α-synuclein in brain. Finally, KuA exerted apparent autophagy enhancement both in vitro and in vivo. In conclusion, KuA protected against neurotoxin-induced PD due to the apoptosis inhibition and autophagy enhancement, suggesting that KuA treatment might represent a neuroprotective treatment for PD.

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1. Introduction

Parkinson’s disease (PD), the second only to Alzheimer’s disease (AD) as the most common age-related neurodegenerative disease, is characterized by the loss of dopaminergic neurons in the substantia nigra (SN) and the presence of Lewy bodies (LBs) composed mainly of α-synuclein [Moore et al., 2005]. The loss of dopaminergic neurons in SN could decrease the level of inhibitory neurotransmitter (dopamine, DA), and in turn destroyed the balance between excitatory neurotransmitter (acetyl choline, ACh) and inhibitory neurotransmitter (DA). Therefore, patients will suffer from different degree of PD symptoms, including muscle rigidity, bradykinesia, and disabling motor abnormalities [Moralesgarcia et al., 2011]. The current drugs, levodopa (L-DOPA), catechol-O-methyltransferase (COMT) inhibitors, monoamine oxidase type B (MAO-B) inhibitors, serotonin agonists [Savitt et al., 2006], could improve the symptoms due to replenishing the absent dopamine (DA), but fail in delaying the disease progression because it could not protect neurons injury [Jankovic and Aguilar, 2008]. Therefore, neuroprotective agents that primarily protect neurons in SN are very important for the progression of PD [Muroyama et al., 2012]. Evidence has demonstrated that protein misfolding and aggregation, disruption of autphagic catabolism, mitochondrial dysfunction, or loss of calcium homeostasis are the main reasons why dopaminergic cells lost in SN [Patrick et al., 2016]. Obviously, agents with single action mechanism may not be adequate in treating a disease with multi-etiology. Thus, a neuroprotective agent with multiple mechanisms may be more beneficial in prevention and treatment of PD.

Cortex Lycii Radicis, a traditional Chinese herb, was generally used as a tonic and reported to exhibit anti-hypotensive, hypoglycaemic, anti-pyretic and anti-stress ulcer activity in animal experiments [Funayama et al., 1980]. Recently, the extracts of Cortex Lycii Radicis was reported to induce autophagy [Wang and Ye, 2016]. Kukoamine A (KuA) (Fig. 1A), a main bioactive ingredient in Cortex Lycii Radicis, has been confirmed to have anti-
hypertension effect in vivo [Lipinski et al., 2001]. Our previous study showed KuA could decrease the oxidative-stress and excitotoxicity [Hu et al., 2015], and could also alleviate the brain damage induced by radiation [Zhang et al., 2016]. These studies suggested that KuA exerted great bioactivity, especially in neuroprotection. However, whether KuA showed the activity of anti-PD and the effects of autophagy induction are still unknown.

In the present study, MPP\(^+\)-induced cell injury model was used to examine the neuroprotective effects of KuA in vitro and MPTP-induced mouse model of PD was used to examine the effects of KuA on PD in vivo. In addition, KuA alone was used to investigate its autophagy enhancement both in vitro and in vivo. All the results indicated that KuA may be a promising agent of PD treatment.

2. Materials and methods

2.1. Chemicals and reagents

Kukoamine A (>98%) was purchased from Chengdu Biopurify Phytochemicals Ltd. (China) (Dissolved in 0.9% NaCl). JC-1 staining kit, BCA proteins determining kit, DAB staining kit, Nissl kit, and caspase-3 activity kit were purchased from Beyotime (Nanjing, China). Lactate Dehydrogenase (LDH) kit and MDC staining kit were purchased from Nanjing Key-Gen Biotech (Nanjing, China). MAO-B detection kits was purchased from Nanjing Jiancheng Biotechnology Institute (Nanjing, China). MTT, 0.25% trypsin-EDTA and dimethyl sulfoxide (DMSO) were purchased from Amresco (Solon, OH, USA). 3-Methyladenine (3-MA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Hoechst 33342, and propidium iodide (PI) were purchased from Invitrogen (Carlsbad, CA, USA). Rabbit antibodies to ERK, p-ERK (p44/p42), AKT, p-AKT, p38, p-p38, JNK, p-JNK, LC-3/I/II, Beclin-1, p62 and TH were purchased from Cell Signalling Technology (Beverly, MA, USA). Rabbit antibody to a-synuclein and mouse antibodies to b-actin, Bax, Bcl-2, cytochrome c and HRP-conjugated goat anti-rabbit (mouse) IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All remaining chemicals and reagents used in this experiment were the highest available analytic grade.

2.2. Cell culture and drug treatments

SH-SY5Y cells were cultured in DMEM-F12 medium with 10% FBS in a humidified atmosphere incubator of 5% CO\(_2\) at 37 °C. The culture medium was changed on alternative day. Then cells were seeded in different kinds of plates with specified density and continued to culture for 24 h. After that, cells were treated with KuA (10 μM, 20 μM, 40 μM) or without KuA or 3-MA (2 mM) for 4 h, and then treated with 1.5 mM of MPP\(^+\) for another 24 h. After these treatments, the supernatant or cells was collected to perform the following experiments.

2.3. Cell viability assay

SH-SY5Y cells were seeded in 96-well plates (5 × 10\(^3\) cells/well) and then were treated as mentioned above (2.2). After these treatments, MTT (final concentration: 0.5 mg/mL) was added to the medium to incubate for 4 h at 37 °C in the dark. Then, the formed insoluble formazan crystals were dissolved with 150 μL DMSO and the formazan dye product was quantified in an ELISA microplate reader (Elx 800 Bio-Tek, USA) at an absorbance of 490 nm. Cell viability was defined as the 100% of control cells.
2.4. Lactate Dehydrogenase (LDH) release assay

The cells were seeded into 96-well plates (5 x 10^4 cells/well) and then were treated as mentioned above (2.2). After these treatments, 20 μL of the supernatant was collected according to the manufacturer’s instructions of LDH assay kit. In accordance with the requirements of the kit, the absorbance at 450 nm was measured by a microplate reader (Elx 800 Bio-Tek, USA).

2.5. Nuclear staining with Hoechst 33342 and PI

Cell death measurements were performed using a fluorometric method under a fluorescence microscope, as described previously [Wrede et al., 2002]. The cells were seeded into 6-well plates (2 x 10^5 cells/well) and then were treated as mentioned above (2.2). After these treatments, Hoechst 33342 (final concentration: 10 μM) and PI (final concentration: 50 μM) were added to incubate for 10 min. Images were obtained using a fluorescence microscope (IX71, Olympus, Japan).

2.6. Determination of Mitochondria Membrane Potential (MMP) with JC-1 probe

MMP was analyzed using a JC-1 staining kit. Briefly, SH-SY5Y cells were seeded into 6-well plates (2 x 10^5 cells/well) and cultured for 24 h. Then KuA (10, 20, 40 μM) were added to the wells to incubate for 4 h. Afterward, the cells were washed for three times with cold PBS buffer. High membrane potential was associated with emission at 530 nm (green). Images were obtained using a fluorescence microscope (IX71, Olympus, Japan).

2.7. Monodansylcadaverin (MDC) staining

The autophagy of SH-SY5Y cells induced by KuA was determined by MDC staining assay. SH-SY5Y cells were seeded into 6-well plates (2 x 10^5 cells/well) and cultured for 24 h. Then KuA (10, 20, 40 μM) were added to the wells to incubate for 4 h. Afterward, the cells were washed for three times with cold PBS buffer. Finally, the cells were incubated with MDC probe (final concentration: 0.05 mM) in the dark at 37°C for 30 min. Then cells were averagely divided into two parts that one was used for taking picture by fluorescence microscope (IX71, Olympus, Japan) and the other one was used for quantitative analysis by FCM flow cytometer (Becton Dickinson, NJ, USA).

2.8. Electron microscope (EM) analysis of SH-SY5Y cells

EM analysis was similar to a previous study [Park et al., 2014]. Cells were seeded into 6-well plates (2 x 10^5 cells/well) to culture for 24 h, and then pretreated with KuA (10, 20, 40 μM) for 4 h. After these treatments, cells were fixed with 2% glutaraldehyde-paraformaldehyde in 0.1 mol/L PB (pH 7.4) for 2 h and washed 3 times for 30 min in 0.1 mol/L PB. Afterward, the cells were postfixed with 1% OsO4 dissolved in 0.1 mol/L PB for 2 h and dehydrated in an ascending gradual ethanol series (50%-100%) and infiltrated with propylene oxide. Specimens were embedded using a Poly/Bed 812 kit (Polysciences, PA, USA). After embedding in pure, fresh resin and polymerization at 60°C in an electron microscope oven (TD-700, DOSAKA, Japan) for 24 h, 350 nm thick sections were initially cut and stained with toluidine blue for viewing under the light microscope. Thin sections (70 nm; LEICA Ultracut UCT Ultramicrotome, Leica Microsystems, Austria) were double stained with 7% (20 min) uranyl acetate and lead citrate for contrast staining. All the thin sections were visualized by transmission electron microscopy (JEM-1011, JEOL, Japan) at an acceleration voltage of 80 kV.

2.9. Animals, drug administration and establishment of PD model

Male C57BL/6 mice (7–8 weeks old, weighing approximately 20–22 g) were obtained from the Experimental Animal Care Center, General Hospital of Shenyang Military Area Command. Animals were housed in a temperature-controlled (22–25°C) room with a 12:12-h light:dark cycle and had free access to food and water. Mice were randomly divided into five groups (five mice/cage), including Group A, control; Group B, KuA-only; Group C, MPTP; Group D, KuA (5 mg/kg) + MPTP (20 mg/kg); Group E, KuA (20 mg/kg) + MPTP (20 mg/kg), and allowed 3 days to acclimate before any treatments. KuA was pre-administered intragastric (i.g.) at 5 mg/kg or 20 mg/kg body weight daily for 12 days. Group A and C animals were administered phosphate-buffered saline (PBS) before MPTP injection. After 12 days of pretreatment with KuA, four times of intra-peritoneally (i.p.) injections of vehicle (PBS) or MPTP (20 mg/kg) were administered to male C57BL/6 mice at 2-h intervals a day [Choi et al., 2012]. All experiments were carried out according to the guidelines of the animal care and use committee at General Hospital of Shenyang Military Area Command.

2.10. Rota-rod test

The test was similar to that described previously [Borlongan et al., 1995] and assessed using a rotary rod apparatus. All mice were pretrained for 3 days prior to drug administration. The training consisted of three consecutive runs with a gradual increase in rpm up to a maximum 30 rpm until the mice were able to keep themselves without falling from the rotary rod for up to 180 s. After MPTP administration, mice were tested at 4, 24, 48, and 72 h. The average time of three tests was calculated for statistical analyses.

2.11. Pole test

Pole test was carried out according to the protocol described previously [Kumar et al., 2013] with slight modifications. The wooden pole (high: 50 cm, diameter: 1 cm) was wrapped in gauze to prevent slipping. A cork ball with diameter of 2.5 cm was glued on top of the pole to position the mice on the pole. The total time the animal took to climb down the pole was measured. If the mouse did not descend in 250 s, the performance was recorded as 250 s. The average time of three tests was calculated for statistical analyses.

2.12. Traction test

Limb impairment was assessed by traction test, as described previously [Arai et al., 1990]. Mice were hung from a horizontal wire by its forepaws. The mouse was scored 3 points if grasped the wire with both hind paws, and 2 points if grasped the wire with one hind paw, and 1 point if not grasped the wire with either hind paw. The average time of three tests was calculated for statistical analyses.

2.13. Assay of MAO-B activity

With the respective detection kits (Nanjing Jiancheng Bio-engineering Institute, China), the activities of MAO-B in the substantia nigra (SN) were determined following the kit instructions. Results were presented as units of activity per mg of protein (wt weight) or content.
2.14. Tissue preparation

At 10 days after MPTP administration, mice were anesthetized with ether for 1 min, and then perfused intracardially with 0.9% sodium chloride (PH = 7.4) and 4% paraformaldehyde (PFA) in 0.1 M sodium dihydrogen phosphate containing 0.48% sodium hydroxide (PH = 7.4). After fixative perfusion, brains were removed, placed in the same fixative solution at 4 °C overnight, and transferred to a 30% sucrose solution till settle down. The cryoprotected brains were sectioned serially at 30 μm in the coronal plane using a freezing microtome (Microm, Walldorf, Germany), collected in Dulbecco’s PBS solution containing 0.1% sodium azide solution, and stored at 4 °C.

2.15. Nissl staining

After three times of washing with PBS, nissl staining was performed according to the instruction book. Briefly, sections were incubated in nissl staining solution for 10 min at 40 °C, and then were washed with different solutions, including water, 95% ethyl alcohol, 70% ethyl alcohol in secceision. Images were obtained with a DP72 digital camera (Olympus, Tokyo, Japan).

2.16. Diaminobenzidine (DAB) immunostaining

DAB immunostaining was similar to a previous study with some slight modifications [Choi et al., 2012]. Briefly, brain sections were treated with 0.6% H2O2 in phosphate-buffered saline (PBS) for 20 min and then washed with Tris-buffered saline (TBS) for three times. After that, sections were blocked in 0.5% Triton-x-100 in TBS and Triton X-100/3% horse serum (TBS-TS) for 60 min at room temperature, and incubated with primary antibody tyrosine hydroxylase (TH) at 1:600 in TBS-TS at 4 °C for 48 h. Then after four times of washing with TBS, sections were incubated with secondary goat-rabbit IgG antibodies (1:1000, Bioworld) at room temperature for 2 h. After three times of washing with TBS, DAB solution was added to incubate for 5 min. Images were obtained with a DP72 digital camera (Olympus, Tokyo, Japan) and DP2-BSW microscope digital camera software (Olympus) and TH-positive cells were counted in entire extents of the SN. All cell counts were performed by the same informed investigator.

2.17. Western-blot analysis

After the experimental treatment, the cells and tissue homogenates were solubilized in RIPA lysis buffer and the protein concentration in each sample was determined using a BCA assay kit. Total protein equivalents for each sample (50 μg protein per lane) were then separated in 12% SDS-polyacrylamide gels and electrophoretically transferred to PVDF transfer membrane. The membranes were immediately placed into blocking solution (5% non-fat milk) at room temperature for 2 h. The membrane was incubated with blocking solution diluted primary antibody for Bax (Rabbit; 1:800), Bcl-2 (Rabbit; 1:800), cytochrome c (Rabbit; 1:800), p-AKT (Rabbit; 1:1000), t-AKT (Rabbit; 1:1000), p-ERK (Rabbit; 1:1000), t-ERK (Rabbit; 1:1000), p-JNK (Rabbit; 1:1000), t-JNK (Rabbit; 1:1000), p-p38 (Rabbit; 1:1000), t-p38 (Rabbit; 1:1000), LC3-A/B (Rabbit; 1:1000), Beclin-1 (Rabbit; 1:1000), p62 (Rabbit; 1:1000), TH (Rabbit; 1:1000), α-synuclein (Mouse; 1:800) and β-actin (Mouse; 1:1000) at 4 °C overnight. After washing three times for 5 min each, the membrane was incubated with secondary anti-rabbit antibody or anti-mouse antibody (1:10000) in TBS-T buffer at room temperature for 1 h. Horseradish-conjugated secondary antibody labeling was detected by enhanced chemiluminescence (ECL) and qualified by densitometric analysis. The results were expressed as the percentage of β-actin (% of control, which was deemed to be 100%).

2.18. Statistical analysis

Data were represented as the mean ± S.E.M. Analysis of variance (ANOVA) with Tukey’s HSD-post hoc test procedure was used to determine the significances of differences between groups. The analysis was performed in Sigmaplot 12.0 software, and P < 0.05 was considered significant.

3. Results

3.1. Effects of KuA on MPP+-induced cell injury

In order to confirm the optimal concentration of MPP+ and the protective effects of KuA on MPP+-induced SH-SY5Y cells injury, various concentrations of MPP+ or KuA were exposed to cells. The results showed that MPP+ could decrease the cell viability in a dose-dependent manner. 1.5 mM of MPP+ could decrease cell viability to 52.72 ± 0.92% (Fig. 1B). However, pretreatment with different concentrations of KuA (10, 20, 40 μM) could significantly increase the cell viability to 71.99 ± 3.21%, 83.94 ± 1.31%, 92.57 ± 1.70%, respectively (Fig. 1C). Further, the results were supported by LDH release (Fig. 1D) that KuA (10, 20, 40 μM) respectively reduced the LDH release to 103.09 ± 3.59 U/L, 72.02 ± 1.71 U/L, 60.99 ± 2.25 U/L compared with MPP+-treated group (124.67 ± 9.35 U/L). Moreover, the morphology of cells was consistent with the mentioned two results (Fig. 2A). These results suggested that KuA had the protective effects on MPP+-induced SH-SY5Y cells injury.

3.2. Effects of KuA on MPP+-induced cell apoptosis

Hoechst-PI double staining was used to evaluate the effects of KuA on apoptosis. As shown in Fig. 2B, DNA condensation (Hoechst 33342-positive cells, blue) and nuclear fragmentation (PI-positive cells, red) were presented in cells which treated with MPP+. However, pretreatment with KuA could reverse the tendency in a dose-dependent manner. These results indicated that KuA had a strong ability of anti-apoptosis that induced by MPP+.

3.3. Effects of KuA on mitochondrial membrane potential (MMP)

In order to investigate the effects of KuA on mitochondrial activity, JC-1 staining was used to analyze the MMP. The fluorescence intensity of high green and low red (low MMP) was presented in the optimal concentration of MPP+-induced SH-SY5Y cells injury, whereas pretreatment with KuA (10, 20, 40 μM) could decrease cell viability to 52.72 ± 0.92% (Fig. 1B). However, pretreatment with different concentrations of KuA (10, 20, 40 μM) could significantly increase the cell viability to 71.99 ± 3.21%, 83.94 ± 1.31%, 92.57 ± 1.70%, respectively (Fig. 1C). Further, the results were supported by LDH release (Fig. 1D) that KuA (10, 20, 40 μM) respectively reduced the LDH release to 103.09 ± 3.59 U/L, 72.02 ± 1.71 U/L, 60.99 ± 2.25 U/L compared with MPP+-treated group (124.67 ± 9.35 U/L). Moreover, the morphology of cells was consistent with the mentioned two results (Fig. 2A). These results suggested that KuA had the protective effects on MPP+-induced SH-SY5Y cells injury.

3.4. KuA inhibited caspase-3 activity and regulated apoptosis-related proteins in MPP+-stimulated SH-SY5Y cells

The mitochondrial apoptosis pathway is involved mainly in the apoptosis of dopaminergic neuronal cells. As shown in Fig. 3A and B, MPP+ increased the expression of Bax and decreased the expression of Bcl-2, resulting in an increase in the Bax/Bcl-2 ratio (3.82 ± 0.06) compared with control group (1.00 ± 0.12), whereas pretreatment with KuA (10, 20, 40 μM) could decrease the Bax/Bcl-2 ratio to 3.77 ± 0.36, 2.22 ± 0.17, 0.68 ± 0.31, respectively. KuA alone didn’t influence Bcl-2/Bax ratio. As shown in Fig. 3A and C, MPP+ increased the release of cytochrome c (5.77 ± 0.24) compared...
with control group (1.00 ± 0.09), whereas pretreatment with KuA (10, 20, 40 μM) could prevent MPP⁺-induced increase of cytochrome c to 3.72 ± 0.25, 1.77 ± 0.08, 1.47 ± 0.03, respectively. KuA alone didn’t influence the release of cytochrome c. These results suggested that KuA could inhibit the mitochondrial apoptosis to prevent cell injury.

3.5. KuA inhibited the expression of p-p38 and p-JNK, and increased the expression of p-ERK and p-AKT

PI3K-AKT signaling pathway and MAPKs family play a key role in MPP⁺-induced cell injury. Western-blot assay was used to investigate the effects of KuA on the expression of these proteins. As shown in Fig. 3D and E, MPP⁺ (1.5 mM) time-dependently decreased the phosphorylation of AKT to 0.45 ± 0.07 compared with control group (relative value = 1). However, pretreatment with KuA (40 μM) could reverse that to 2.05 ± 0.07. In addition, the phosphorylation of ERK had a similar tendency with p-AKT. KuA (40 μM) could obviously increase the expression of p-ERK (3.31 ± 0.14) compared with MPP⁺-treated group (2.21 ± 0.07) (Fig. 3D and F). Furthermore, as shown in Fig. 3D and G, MPP⁺ (1.5 mM) time-dependently increased the phosphorylation of JNK (1.89 ± 0.07) compared with control group (relative value = 1). However, pretreatment with KuA (40 μM) could reverse that to 0.57 ± 0.02. Moreover, MPP⁺ (1.5 mM) time-dependently decreased the phosphorylation of p38 (1.53 ± 0.17) compared with control group (relative value = 1). However, pretreatment with KuA (40 μM) could reverse that to 0.21 ± 0.06 (Fig. 5D and H). These results suggested that KuA could regulate MAPKs and AKT to provide its neuroprotection.

3.6. KuA induced autophagy in vitro

MDC staining and EM analysis were employed to investigate whether KuA could induce autophagy. As shown in Fig. 4A, the fluorescence intensity of SH-SY5Y cells stained with MDC probe was increased by KuA in a dose-dependent manner. And the FCM results showed that KuA (10, 20, 40 μM) could respectively increase the fluorescence intensity of SH-SY5Y cells to 121.80 ± 6.81%, 147.48 ± 3.13%, 205.60 ± 6.29% compared with control group (100.00 ± 6.81%) (Fig. 4B). In addition, picture of EM analysis showed apparent autophagosome which possessed dual membrane structure at the concentration of 40 μM (Fig. 4C). These results suggested that KuA could lead to autophagy enhancement in SH-SY5Y cells.

3.7. The protective effect of KuA against MPP⁺ was inhibited by 3-MA

In order to verify that the anti-PD effect of KuA was partially related to autophagy, MTT assay was used to detect whether 3-MA, an autophagic inhibitor, could decrease the protective effect of KuA on cells. As shown in Fig. 4D, 2 mM of 3-MA could weaken the protective effect of KuA on cell viability compared with KuA alone. These results suggested that KuA provided protective effect against MPP⁺ partially through autophagic pathway.
3.8. KuA regulated autophagy-related proteins both in vitro and in vivo

The expression of autophagy-related proteins were changed as autophagy enhancement. As shown in Fig. 5A, KuA (10, 20, 40 μM) respectively increased LC3-II/LC-I ratio to 1.23 ± 0.09, 1.96 ± 0.08, 2.12 ± 0.09 compared with control (1.00 ± 0.99). KuA (10, 20, 40 μM) respectively increase Beclin-1 to 1.33 ± 0.65, 2.21 ± 0.16, 3.27 ± 0.09 compared with control (1.00 ± 0.12). KuA (10, 20, 40 μM) respectively decreased p62 to 0.79 ± 0.05, 0.48 ± 0.03, 0.33 ± 0.02 compared with control (1.00 ± 0.04). In addition, KuA could also regulate these proteins in a time-dependent manner. As shown in Fig. 5B, 40 μM of KuA (1, 2, 3, 4 h) could respectively increased LC3-II/LC-I ratio to 1.34 ± 0.04, 2.13 ± 0.11, 2.53 ± 0.10, 3.32 ± 0.11 compared with control group (1.00 ± 0.17). 40 μM of KuA (1, 2, 3, 4 h) could respectively increase Beclin-1 to 1.82 ± 0.07, 2.28 ± 0.12, 2.77 ± 0.07, 3.16 ± 0.04 compared with control group (1.00 ± 0.13). 40 μM of KuA (1, 2, 3, 4 h) could respectively decrease p62 to 0.81 ± 0.03, 0.63 ± 0.02, 0.41 ± 0.01, 0.25 ± 0.02 compared with control group (1.00 ± 0.04). Moreover, KuA could increase the expression of LC3-II/LC-I ratio that was decreased by 3-MA, and KuA could also decrease the expression of p62 that was increased by 3-MA (Fig. 5C). In order to verify if KuA could regulate these proteins in vivo, C57BL-6 mouse was used to study the expression of LC3-II/LC-I ratio, Beclin-1 and p62 in SN and Str. The results showed that pretreatment with KuA for 12 days (Fig. 6A) could upregulate the expression of LC3-II/LC-I ratio and Beclin-1, and downregulate the expression of p62 in vivo (Fig. 6B and C). These results suggested that KuA could regulate proteins that related to autophagy.

3.9. KuA improved the dyskinesia induced by MPTP

To investigate the effects of KuA on motor function, three kinds of behavior tests, including rota-rod test, pole test and traction test were conducted in our study. All mice were pretrained for 3 days to ensure themselves to adapt the devices (Fig. 7A). The body weight of each mouse was recorded during the drug treatment (Fig. 7B). Rota-rod test showed that the MPTP significantly impaired performance (##P < 0.01) compared with normal controls at 4, 24, 48, and 72 h after the last MPTP injection. However, KuA at 5 mg/kg and 20 mg/kg significantly alleviated the impairment (##P < 0.01) obtained in the MPTP-treated group at 6, 24, 48, and 72 h after MPTP injection (Fig. 7C). Traction test showed that MPTP significantly decreased limb movements scored (##P < 0.01) compared with controls. However, pretreatment with KuA at 5 mg/kg and 20 mg/kg significantly increased the limb movements scored (##P < 0.01).
3.10. KuA could prevent neuronal loss and α-synuclein level induced by MPTP in SN and Str

In order to confirm the effects of KuA on neuronal activity and α-synuclein level, nissl staining and western-blot analysis were used to detect the level of nissl substance and apoptosis-related proteins in SN and Str. As shown in Fig. 8A, MPTP could lead to the decrease of nissl substance that was dyed purple compared with control group and KuA-alone group in SN and Str. However, pretreatment with KuA (5 mg/kg and 20 mg/kg) for 12 days could significantly increase the level of nissl substance. In addition, the above results were supported by western-blot analysis of apoptosis-related proteins that MPTP could lead to the increase the expression of Bax and cytochrome c and the activity of caspase-3, and decrease the expression of Bcl-2 in SN and Str. However, pretreatment with KuA (5 mg/kg and 20 mg/kg) for 12 days could significantly reverse this tendency in a dose-dependent manner (Fig. 8B, C and D). Moreover, MPTP could induce the increase of α-synuclein level compared with control group and KuA-alone group. However, pretreatment with KuA (5 mg/kg and 20 mg/kg) for 12 days could significantly decrease the level of α-synuclein in a dose-dependent manner (Fig. 8B and C). These results suggested that KuA could protect neuronal in SN and Str.

3.11. KuA attenuated dopaminergic neuronal loss in SN and Str in the MPTP-induced mouse model

To observe the neuroprotective effects of KuA, Str and SN sections were immunostained with TH (a marker of dopaminergic neurons). The MPTP group showed significantly fewer TH-positive cells than normal group and KuA-alone group in SN and Str. However, pretreatment with KuA at 5 mg/kg and 20 mg/kg could significantly prevent this loss in Str (Fig. 9A) and SN (Fig. 9B). To confirm these results, the expression of TH protein in SN (Fig. 9C) and Str (Fig. 9D) was detected by western-blot analysis. The results showed that TH protein levels were significantly lower in the MPTP group than in normal controls and KuA-alone group, and pretreatment with KuA (5 mg/kg and 20 mg/kg) could attenuate MPTP-induced TH loss. These results showed that KuA protected against MPTP-induced dopaminergic neuronal loss.

3.12. Effects of KuA on the activity of MAO-B

In order to investigate whether KuA could influence the translation of MPTP to MPP⁺, MAO-B activity was detected. The results showed that MPTP could increase the MAO-B activity to 65.34 ± 3.53 U/mg compared with control group (38.45 ± 3.42 U/mg). However, pretreatment with KuA (5 mg/kg, 20 mg/kg) didn’t influence the activity of MAO-B (Fig. 10). The results indicated that KuA could not change the activity of MAO-B.

4. Discussion

Several studies have indicated that α-synuclein misfolding and overexpression, disruption of autophagic catabolism, mitochondrial dysfunction, and loss of calcium homeostasis are intimately linked with the pathogenesis of PD [Patrick et al., 2016]. Our
Fig. 5. The effects of KuA on autophagy-related proteins in vitro. (A) The original bands and density quantitative of LC3, Beclin-1 and p62 with dose-dependent manner. (B) The original bands and density quantitative of LC3, Beclin-1 and p62 with time-dependent manner. (C) The original bands and density quantitative of KuA and 3-MA on the expression of LC3, Beclin-1. Data were presented as Means ± S.E.M. (n = 3). \#p < 0.05 and \##p < 0.01 compared with control group. *p < 0.05 and **p < 0.01 compared with MPP⁺-treated group.

Fig. 6. The effects of KuA on the expression of autophagy proteins in vivo. (A) The flow chart of experiments. (B) The original bands and density quantitative of LC3, Beclin-1 and p62 in SN. (C) The original bands and density quantitative of LC3, Beclin-1 and p62 in Str. All the results were randomly obtained from three of the five mice/group.
previous study demonstrated that KuA could provide neuroprotective effects through mitochondrial pathway, calcium antagonism [Hu et al., 2015], and inflammation pathway [Zhang et al., 2016]. All of these reminded that KuA might have anti-PD bioactivity. In the present study, thus, we investigated the anti-PD bioactivity of KuA and its potential mechanisms.

MPTP has been the most valuable neurotoxin for inducing PD model of animals [Langston et al., 1983]. MPP\(^+\), the ion pair form of MPTP, was formed through MPTP oxidation by monoamine oxidase-B (MAO-B) in vivo [Shenkar et al., 1990], resulting in the injury of dopaminergic neuron [20]. Therefore, MPP\(^+\)-induced cell injury model and MPTP-induced dopaminergic neuron loss in vivo were used in our present study to simulate PD. Our results showed that pretreatment with KuA didn’t influence MAO-B activity, the results indicated that KuA didn’t influence the translation of MPTP to MPP\(^+\). As mentioned in the introduction, the damaged dopaminergic neurons of SN make DA deficiency, which makes patients suffer from different degrees of behavior disorders. Therefore, the motor function is commonly used as an evaluation index of PD. Rota-rod test, pole test, and traction test were used to evaluate the effects of agent on behavior [Kumar et al., 2013; Arai et al., 1990; Langston et al., 1983]. Our results showed that KuA effectively increased the duration time of mice on rotating-stick, and increased the grasping force as well as the rate of climbing pole compared with MPTP-treated group. These results suggested that KuA might be a potential anti-PD agent due to its improvement of motor function.

In order to investigate whether KuA could directly protect against MPP\(^+\)-induced cytotoxicity, we used an in vitro system human neuroblastoma SH-SY5Y cells. Our results showed that KuA could effectively protect against MPP\(^+\)-induced cell injury (Figs. 1 and 2). More important, nissl staining showed that KuA could increase the neuronal activity of SN (Fig. 6A). These results showed that KuA directly protected neuronal both in vitro and in vivo. To further demonstrated the effects of KuA on PD, immunohistochemistry (IHC) analysis and western-blot analysis for tyrosine hydroxylase (TH) protein were conducted. The early loss of tyrosine hydroxylase (TH) activity followed by a decline in TH protein levels is thought to contribute to dopamine deficiency. Because the decrease of TH-positive neurons in response to MPTP treatment is the most prominent at medial levels of the SN. Our results showed that KuA could increase TH-positive cells in SN and Str compared with MPTP treatment group and KuA could also increase the expression of TH both in SN and Str (Fig. 9). All the results suggested that KuA could be a promising agent of PD treatment.

The neuronal loss in the SN, which may lead to dopamine deficiency, partly due to the accumulation of aggregated/misfolded proteins [Patrick et al., 2016]. Most aggregated/misfolded proteins are cleaned via two important pathways: the ubiquitin proteasome system (UPS) and the autophagy-lysosome pathway (ALP) [Klionsky and Emr, 2000]. Evidences have shown that autophagic pathway is default pathway and becomes increasingly important in PD. Because the PD induced by misfolded \(\alpha\)-synuclein could be cleared by autophagic pathway [Bandhyopadhyay and Cuervo, 2007], which supports the hypothesis that impairing the autophagic degradation will aggravate the progress of PD [Cuervo et al., 2004]. Our results showed that KuA could decrease the \(\alpha\)-synuclein expression both in SN and Str (Fig. 8). In addition, a previous study had demonstrated that the extracts of Cortex Lycii Radicis to have autophagy induction [Wang and Ye, 2016], we easily speculated that KuA, the main bioactive ingredient in Cortex Lycii Radicis, might also have autophagy induction. To our delight, the
results showed that treatment with KuA could increase the fluorescence intensity of the cells stained with MDC probe (Fig. 4A and B), and autophagosome could be seen after KuA treatment (Fig. 4C). The induction of autophagy of KuA was further assessed by detecting protein light chain 3 (LC3), an autophagy-related gene 8 (Atg8), which is considered as a specific marker of autophagy. LC3 contains two forms, LC3-I and LC3-II. The expression ratio of LC3-II/LC3-I correlates with the number of autophagosomes [Kabeya et al., 2000]. Our results showed that KuA could increase the expression of LC3-II in time- and dose-dependent manner in cellular model (Fig. 5A). In addition, we detected the expression of Beclin-1, a component of the phosphatidylinositol-3-kinase complex, which is required for autophagy [Kabeya et al., 2000], as another marker of autophagy. Our results showed that KuA could increase the expression of Beclin-1 in a time- and dose-dependent manner in cellular model (Fig. 5B). The expression of these proteins in vivo (Fig. 6) consisted with that in vitro. Last but not least, 3-methyladenine (3-MA), an inhibitor of autophagy, could decrease the protective effect of KuA on cell injury (Fig. 4D). All the mentioned results suggested that KuA could induce autophagy both in vitro and in vivo and the effect of KuA on PD was partly via autophagy enhancement.

5. Conclusion

In summary, it was the first time that we found KuA to have autophagy enhancement both in vitro and in vivo. In addition, our findings indicated that KuA protected against MPP+-induced injury in SH-SYSY cells, and protected against MPTP-induced PD model of mouse. Taken present study together, we concluded that the mechanisms of anti-PD of KuA appeared to be associated with autophagy enhancement, α-synuclein degradation and anti-
apoptosis.

Conflict of interest

Neither any of the authors have anything to disclose regarding this manuscript nor do they have any potential conflicts of interest to report concerning this article.

Acknowledgment

This work was supported by the National Science and Technology Major Project, People’s republic of China. (Project number: 2014ZX09J14101-05C).

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