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Baicalein exerts neuroprotective effects in 6-hydroxydopamine-induced experimental parkinsonism in vivo and in vitro

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ABSTRACT

Baicalein, a flavonoid obtained from the root of Chinese medicinal herb *Scutellaria baicalensis*, has been shown to exert a protective effect on neurons against several neuronal insults. The aim of this study was to explore the neuroprotective effect of baicalein in 6-hydroxydopamine (6-OHDA)-induced experimental parkinsonism in vitro and in vivo. In in vitro experiments, we found that baicalein (0.5, 5 μg/mL) could significantly ameliorate the 6-OHDA-induced SH-SY5Y cell apoptosis from 31.56% in the 6-OHDA group to 18.90%, 21.61% respectively, and also promote neurite outgrowth of PC12 cell. In in vivo experiments, baicalein had no effect on apomorphine (APO)-induced rotations, but it could significantly attenuate muscle tremor of 6-OHDA-lesioned rats. The burst frequency and amplitude are 13.43%, 35.18% compared to 6-OHDA group. Moreover, baicalein treatment could also increase tyrosine hydroxylase (TH)-positive neurons to 265.52% of the 6-OHDA group. The neuroprotective action of baicalein was coincident with an attenuated astroglial response within the substantia nigra. Neuroprotective effect of baicalein as demonstrated by the increasing the number of dopaminergic neurons may have been, in part, caused by anti-apoptotic, pro-differentiation and anti-inflammatory mechanisms of baicalein. Therefore, baicalein can be a promising candidate for prevention or treatment of Parkinson's disease, owing to its anti-apoptotic, pro-differentiation and anti-inflammatory action.

1. Introduction

Parkinson's disease (PD) is a typical neurodegenerative disorder, characterized by symptoms including rest tremors, postural instability, gait abnormality, bradykinesia and rigidity. The major pathological change of Parkinson's disease is the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Olanow and Tatton, 1999). Neurotoxin to dopaminergic neurons such as 6-hydroxydopamine (6-OHDA), are usually applied to induce experimental parkinsonism. 6-OHDA is a dopamine analog, which can be uptaken into catecholaminergic nerve endings and induces death of dopaminergic neurons. 6-OHDA has been reported to produce some of the behavioural, biochemical, and pathological changes that are encountered in Parkinson's disease (Schober, 2004). The toxic effects of 6-OHDA are attributed to the formation of free radicals, inflammatory processes and apoptosis (Ikeda et al., 2008; Kobayashi et al., 2008; Koprich et al., 2008; Kumar et al., 1995; Perumal et al., 1992).

Baicalein is one of the major flavonoids originally isolated from the roots of the traditional Chinese herbal medicine Huangqin, *Scutellaria baicalensis* Georgi. Baicalein is a potent antioxidant and free radical scavenger, and has been regarded as the 12/15-lipoxygenase inhibitor and xanthine oxidase inhibitor (van Leyen et al., 2006). Baicalein also has anti-inflammatory properties since it has been shown to antagonize the expression of adhesion molecule induced by interleukin-β1 (IL-β1) and tumor necrosis factor (TNF-α) (Hsieh et al., 2007). Recent studies have shown that baicalein had neuron-protection against amnesia induced by β-amyloid peptide-(25–35) (Lebeau et al., 2001; Wang et al., 2004) and neuronal injury secondary to ischemia insult (Hwang et al., 2002; Lee et al., 2003). These pharmacologic properties suggest that baicalein may be a useful agent for prevention or treatment of neurodegenerative diseases such as Parkinson's disease.

Therefore, we speculate that baicalein may block 6-OHDA-induced neurotoxicity and prevent degeneration of dopaminergic neurons. The purpose of this study is to investigate the effects of baicalein on 6-OHDA-induced neurotoxicity in vitro and in vivo.

2. Materials and methods

2.1. Drugs and reagents

Baicalein was purchased from Mian yang high-tech Dongfangyuan bio-technology co. Ltd. The purity of Baicalein is 98% tested by HPLC method. 6-OHDA hydrobromide, apomorphine hydrochloride and nerve growth factor were purchased from Sigma-Aldrich.

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2.2. In vitro assay

2.2.1. Cell culture

Human neuroblastoma cells (SH-SY5Y) and PC12 cells were purchased from the Cell Center of the Institute of Basic Medical Science Research (Chinese Academy of Medical Sciences, China). SH-SY5Y cells were cultured in DMEM: F12 (1:1) supplemented with 10% FBS, penicillin (100 IU/mL) and streptomycin (100 μg/mL); PC12 cells, originated from rat pheochromocytoma, were maintained in RMPI-1640 containing 5% FBS and 10% horse serum. The medium was replaced every 2 days. Cell cultures were maintained at 37 °C in a humidified atmosphere containing 95% air: 5% CO_2.

2.2.2. Drug treatments

SH-SY5Y cells were split in 96 well plates at a density of 1.0×10^4/well, and treated with different concentrations of baicalein (0.05, 0.5, 5 μg/mL) for 1 h, then exposed to 100 μM 6-OHDA for 24 h in the presence of baicalein. PC12 cells were seeded at a density of 3.0×10^4/well in 96 well plates and treated with different concentration of baicalein (0.05, 0.5, 5 μg/mL) or nerve growth factor (NGF) (50 ng/mL) for 1 h, then exposed to 100 μM 6-OHDA for 24 h in the presence of baicalein or NGF. NGF served as a positive control.

2.2.3. Cell viability assay in SH-SY5Y cells

We measured cell viability using a luciferase-coupled ATP quantification assay (CellTiter-Glo; Promega, Madison, WI) in accordance with the manufacture's directions. In this assay, luminescent signal is proportional to the amount of ATP. Cell injury and death result in a dramatic decrease in intracellular ATP levels. The results were expressed as the relative luminescence units (RLU). All assays were performed three times or more.

2.2.4. Hoechst 33258 stainings in SH-SY5Y cells

After 6-OHDA challenged for 24 h, nuclear staining of SH-SY5Y cells was performed with Hoechst 33258 (1 mg/mL in PBS). Fluorescence was observed using an inverted fluorescence microscope (Olympus IX71) to distinguish the apoptotic cells by their fragmented and condensed nucleus.

2.2.5. Flow cytometry assay in SH-SY5Y cells

After treatment, 5×10^6 cells were trypsinized, washed twice with PBS, centrifuged at 1000 r/min for 5 min, and then fixed with 70% ethanol overnight at 4 °C. The cells were centrifuged and washed twice with PBS, resuspended in 0.5 mL PBS (containing 50 μg/mL RNaseA), incubated for 30 min at 37 °C. The samples were stained with PI (50 μg/mL) for 30 min at room temperature in the dark, then read in a FACScalibur flow cytometer (USA) at 488 nm excitation. A 600 nm bandpass filter for PI detection was used. Analyses were performed by the software supplied in the instrument.

2.2.6. Cell viability and morphology assay in PC12 cells

At the setting point, PC12 cells were visualized by phase-contrast microscopy using an inverted microscope (Olympus IX71) connected to a digital camera. The cell viability was determined by MTT method.

2.3. In vivo assay

2.3.1. Animals

Adult male Sprague–Dawley rats (180–200 g; Beijing Vital River Laboratory animal technology Co., Ltd; licence: SCXK (JING) 2007-0001) were used in this study. All experiments were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by our local Animal Ethics Committee.

2.3.2. Surgery

Rats were anaesthetised with 3% sodium pentobarbital (45 mg/kg i.p.) and received unilateral lesions of the left medial forebrain bundle (MFB) made by stereotaxic injection of 6-OHDA. 6-OHDA was dissolved in sterile 0.01% ascorbate saline at a concentration of 4 μg/μL and was injected unilaterally (0.5 μL/min) in two deposits (2.5 μL and 3 μL, respectively) at the following coordinates according to the atlas of Paxinos and Watson (1986) (in mm relative to bregma and the surface of the dura mater): anterior (A) = −4.0, lateral (L) = 0.8, ventral (V) = −8.0, tooth bar at +3.4; and A = −4.4, L = 1.2, V = −7.8, tooth bar at −2.4, respectively. The sham-lesioned rats ($n=12$) received only vehicle at the same coordinates.

2.3.3. Apomorphine-induced rotation

The rotation of all rats induced by apomorphine (0.5 mg/kg, s.c.) was tested at 2 weeks after 6-OHDA lesion. Rats were placed in individual transparent cylinder with a diameter of 20 cm. They were allowed to habituate to their environment for 10 min before the administration of apomorphine. Full 360° turns in the direction contralateral to the lesion were counted, and rotational behaviors were assessed for 40 min. Results were expressed as contralateral turns/40 min.

2.3.4. Drug administration procedures

Thirty-six rats which showed more than 280 rotations per 40 min (turns contralateral to the lesion subtracted from ipsilateral turns) were used for further investigation. The selected rats were divided into three groups randomly according to rotation number: Group A, sham-lesioned rats (control, saline i.g.); Group B (model, saline i.g.); Group C (baicalein 200 mg/kg i.g.) and Group D (madopar 50 mg/kg i.g.). All the rats were treated as described above since the 15th day post-lesion. And at day 21, 28, 35 post-lesion, the rotational behaviors were tested.

2.3.5. Monitoring of 6-OHDA-induced tremors

The tremor recording apparatus (BL-420S, Tme, Chengdu, China) consisted of a sensor assembly that generates waves with amplitudes and frequencies determined by the forces and the rate of the movements generating the forces, the interface unit with gain and baseline adjustments, and an IBM compatible personal computer. This apparatus records and stores the activity profile from the sensor assembly in a continuous, time-dependent manner for subsequent

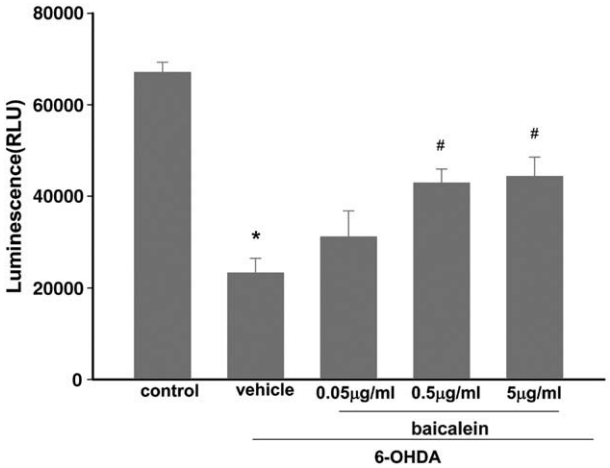

Fig. 1. Protective effects of baicalein on 6-OHDA-treated SH-SY5Y cells. SH-SY5Y cells were treated with 100 μM 6-OHDA for 24 h in the presence or absence of baicalein. These results were expressed as RLU. Data were expressed as mean ± SEM of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ compared with control group, $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with 6-OHDA group.

analysis. After baicalein treatment for one week, rat was put in a cage that was then placed on the sensor. The recording parameters were set according to the manufacturer's instructions by adjusting the Gain, which ensured that most of the signals with large or small amplitudes remained within the detection limit or not reduced to the baseline respectively, as viewed on the computer monitoring screen. The noise that could interfere with the 6-OHDA-induced tremors is usually of low amplitude, and was reduced to baseline levels by this gain setting. This setting was used for subsequent recording and storage of the vibration signals due to tremor-like activities of the 6-OHDA-injected animals as well as the random movement activities of the control animals. The burst amplitude and burst frequency were determined for each rat.

2.3.6. Immunohistochemistry

For immunohistochemical study, six of the rats were perfusion-fixed with 4% paraformaldehyde following a heparinized saline flush 7 days after behavioral assessment. Brains were dissected and postfixed in paraformaldehyde overnight at 4 °C, after which they were transferred into 30% sucrose in 0.1 M PB at 4 °C for 24 h. Then using a cryostat, series of 30 μm thick coronal sections were cut through the ventral mesencephalon. Nigral brain sections were rinsed

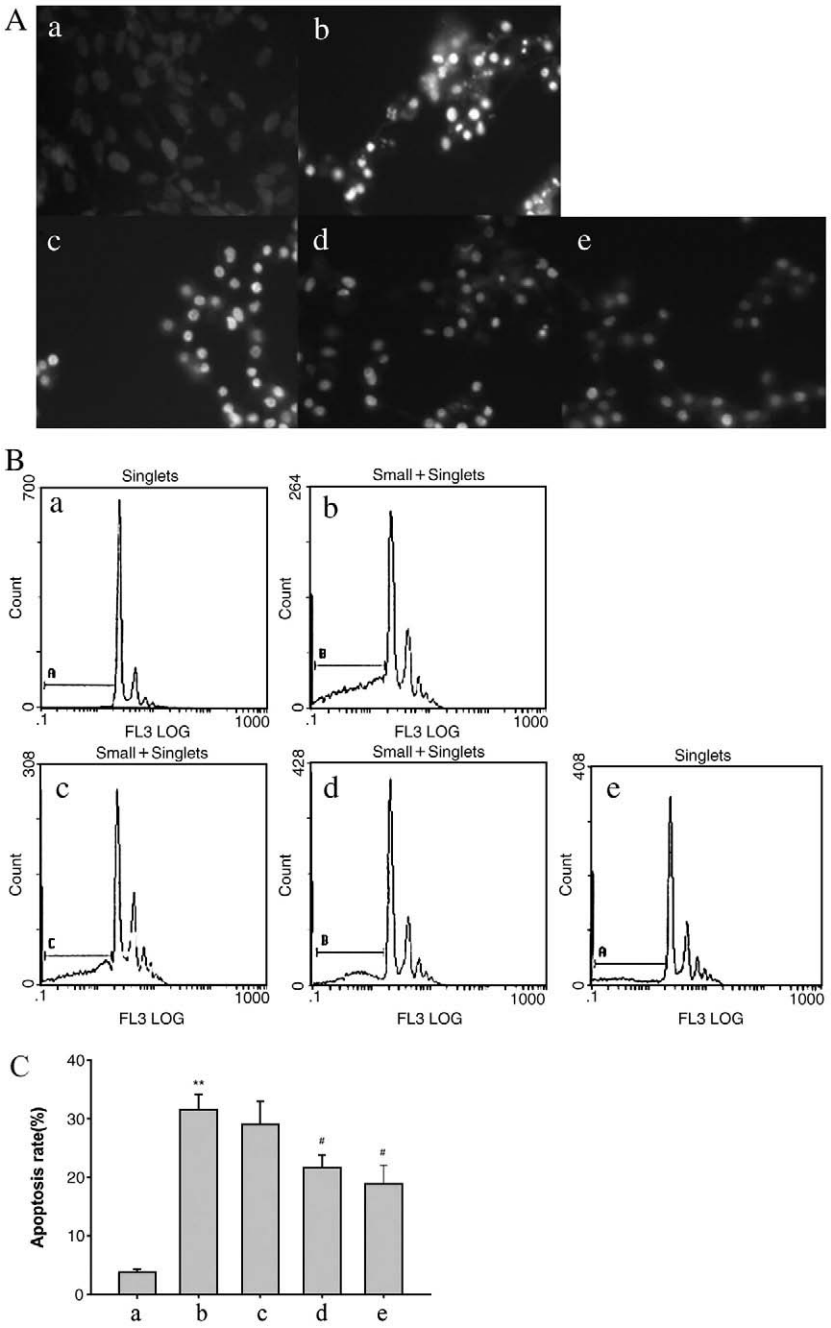

Fig. 2. Effect of baicalein on the 6-OHDA-induced SH-SY5Y cells apoptosis. A: Morphological analysis of nuclear chromatin by Hoechst 33258 staining. Representative photographs showing control (a), 6-OHDA + vehicle (b), 6-OHDA + baicalein(0.05 μg/mL) (c), 6-OHDA + baicalein(0.5 μg/mL) (d), 6-OHDA + baicalein(5 μg/mL) (e). The number of apoptotic 6-OHDA-treated cells decreased by the treatment with 0.5 and 5 μg/mL baicalein (d and e), compared to that without baicalein (b). B and C: Quantitative determination of apoptosis rate by flow cytometric analysis. $^{*}P<0.05$, $^{**}P<0.01$ compared with control group, $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with 6-OHDA group.

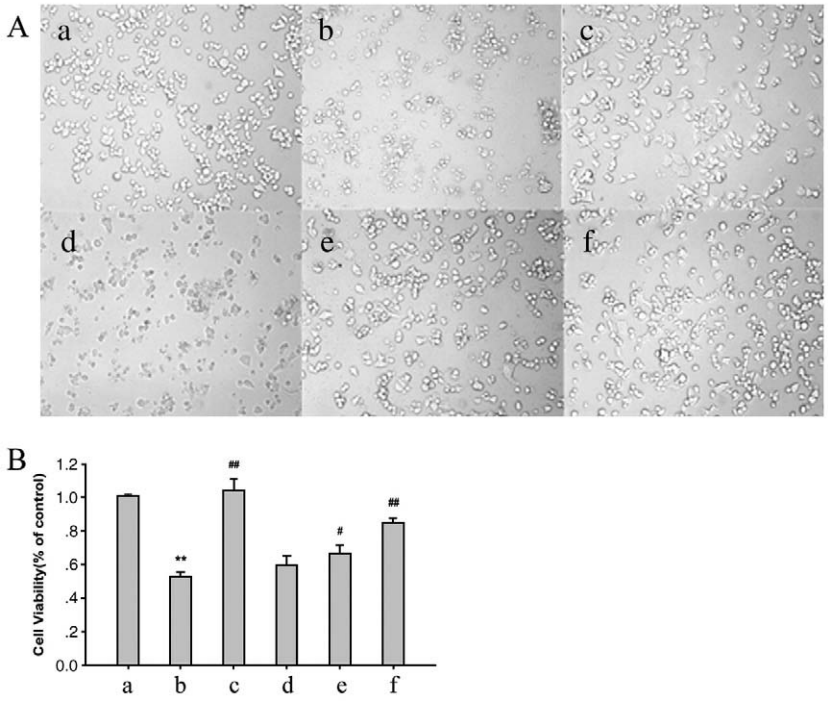

Fig. 3. Protective effects of baicalein against 6-OHDA-induced PC12 cells injury. A: Morphological analysis of PC12 cells by phase-contrast microscopy. Representative photographs showing control (a), 6-OHDA + vehicle (b), 6-OHDA + NGF (c), 6-OHDA + baicalein (0.05 μg/mL) (d), 6-OHDA + baicalein (0.5 μg/mL) (e), 6-OHDA + baicalein (5 μg/mL) (f). B: Determination of cell viability by MTT method. $^{*}P<0.05$, $^{**}P<0.01$ compared with control group, $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with 6-OHDA group.

in PBS + Triton X-100 (PBST), quenched in 3% H_2O_2 and then incubated in blocking solution. After incubated with the anti-tyrosine hydroxylase (TH, monoclonal mouse, Chemicon, 1:500) and anti-glial fibrillary acidic protein (GFAP, polyclonal rabbit, Chemicon, 1:1000) at 4 °C overnight, the sections were treated with biotinylated secondary antibody for 1 h at 37 °C, then with streptavidin-peroxidase for 1 h. Subsequently the sections were incubated with 3, 4-diaminobenzidine. The results were analyzed by counting the numbers of positive cells at ×200 magnifications on an Olympus microscope (1X-70, Olympus Corp., Japan). The average number of positive cells was used to represent cell density.

2.4. Statistical analysis

Values were expressed as means ± SEM. To analyze the differences between groups, statistical analysis was conducted with one-way ANOVA tests followed by Dunnett's test. A P value <0.05 was considered significant.

3. Results

3.1. In vitro assay

3.1.1. Effect of baicalein on cell viability in SH-SY5Y cells

Cell viability assay showed that 100 μM 6-OHDA decreased the cell counts to 36% of control, while pretreatment with baicalein (5, 0.5 μg/mL) attenuated the 6-OHDA-induced cytotoxicity to a large extent (66% and 64% of control respectively Fig. 1).

3.1.2. Effect of baicalein against 6-OHDA-induced apoptosis in SH-SY5Y cells

Typical photographs of Hoechst 33258 stainings in SH-SY5Y cells are shown in Fig. 2A. Normal control cells displayed normal nuclear morphology with a weak fluorescence intensity. Treatment with 6-OHDA at 100 μM for 24 h induced apoptotic cell death accompanied by nuclear condensation and/or fragmentation. Pretreatment with baicalein at 5 μg/mL displayed a significant decrease of apoptotic cells induced by 6-OHDA. To further confirm the effect of baicalein against 6-OHDA-induced apoptosis, we stained SH-SY5Y cells with PI dye and counted the stained cells with flow cytometry. As shown in Fig. 2B and C, the 6-OHDA at 100 μM induced significant cell death, the percentage of apoptotic cells being 31.56 ± 2.58%, against 3.81 ± 0.51% in the control group. Pretreatment with baicalein (5, 0.5 μg/mL) significantly inhibited this cell death, the percentage of apoptotic cells being 18.90 ± 3.17%, 21.61 ± 2.16%, respectively.

3.1.3. Effects of baicalein on cell viability and morphology in PC12 cells

As shown in Fig. 3B, 100 μM 6-OHDA can significantly decrease the PC12 cell counts to 53% of control. Interestingly, NGF (50 ng/mL) can completely abolish this toxicity. And baicalein (5 and 0.5 μg/mL) attenuated 6-OHDA-induced neurotoxicity to a large extent (85% and 67% of control respectively). At the same time, pretreatment with baicalein (5 and 0.5 μg/mL) leads to 6-OHDA-lesioned PC12 cells

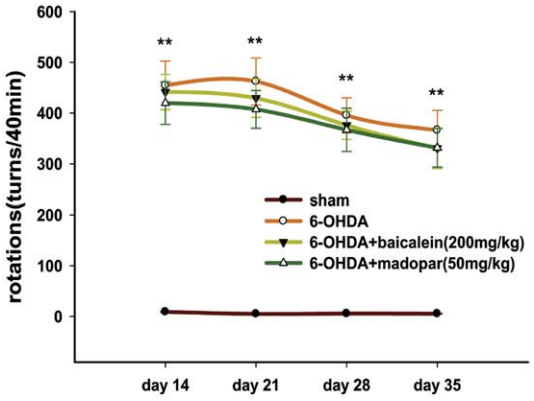

Fig. 4. Effect of baicalein on rotational behavior in 6-OHDA-lesioned rats. Values are expressed as mean ± SEM ($n = 12$). $^{*}P<0.05$, $^{**}P<0.01$ compared with sham group, $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with 6-OHDA group.

acquiring polygonal shapes, which was similar to the effect of NGF (Fig. 3A).

3.2. In vivo assay

3.2.1. Effect of baicalein on apomorphine-induced rotation in 6-OHDA unilaterally lesioned rats

As shown in Fig. 4, rats exhibited rotational behavior in the direction opposite to the lesion (contralateral rotation) following apomorphine challenge 2 weeks after unilateral administration of 6-OHDA. Significant increases in the number of apomorphine-induced rotations were seen in 6-OHDA-lesioned rats compared with the sham-operated rats ($P<0.01$). However, baicalein treatment has no effect on apomorphine-induced rotations ($P>0.05$).

3.2.2. Effect of baicalein on tremor in 6-OHDA unilaterally lesioned rats

As shown in Fig. 5A, the recordings revealed characteristic of muscle activity in sham-operated rats, but 6-OHDA unilaterally lesioned rats showed a high burst activity. However both baicalein and madopar can prevent the burst activity. At the same time, we analyzed the burst frequency and amplitude, the results showed that 6-OHDA could significantly augment the burst frequency and burst amplitude compared with controls ($P<0.01$). Baicalein (200 mg/kg) could decline the burst frequency and burst amplitude to a significant extent ($P<0.01$, Fig. 5B and C). The burst frequency and amplitude are 13.43%, 35.18% compared to 6-OHDA group.

3.2.3. Effects of baicalein on TH and GFAP immunostainings

Representative microphotographs of TH and GFAP immunostainings in the substantia nigra are shown in Fig. 6A. The TH-immunoreactive neurons were easily detectable in the substantia nigra of control rats. The bodies and fibers of dopaminergic cells showed intense staining with evident immunopositive processes. In the 6-OHDA-lesioned rats, few TH-immunopositive neurons were observed which decreased to 8% of the sham rats ($P<0.01$). Baicalein treatment (200 mg/kg) can significantly increase TH-immunopositive neurons to 265.52% of the 6-OHDA group, which is similar to madopar ($P<0.05$, Fig. 6B). GFAP-immunoreactive astrocytes were absent in the substantia nigra of control rats. However, the counts of GFAP-immunopositive astrocytes were markedly increased in 6-OHDA-lesioned group ($P<0.01$). And baicalein can significantly ameliorate the severe increase of GFAP immunoreactivity ($P<0.01$, Fig. 6B).

4. Discussion

The selective loss of dopaminergic neurons in the substantia nigra appears to be the direct cause of neurodegeneration in cases of Parkinson's disease (Olanow and Tatton, 1999). Also, 6-OHDA, which is commonly used for the induction of Parkinson's disease in experimental animals, is believed to cause dopaminergic cell death (Schober, 2004). Baicalein, a flavonoid extracted from a traditional Chinese herbal *S. baicalensis* Georgi (Huangqin), exerts a protective role on neurons. The aim of the present study was to evaluate the neuroprotective potential of baicalein on 6-OHDA-induced dopaminergic neuronal damage in vitro and in vivo.

To examine the neuroprotective properties of baicalein in cultured cells, SH-SY5Y and PC12 cells were used in this study. SH-SY5Y cell is a dopaminergic cell line and has the same characteristics as Parkinson's disease. In the present SH-SY5Y cell assay, 6-OHDA-mediated toxicity exhibited the morphological characteristics of apoptosis, such as nuclear condensation and cell shrinkage, while baicalein potently attenuated the cell damage and could prevent SH-SY5Y cells apoptosis induced by 6-OHDA. Rat pheochromocytoma (PC12) cell line, which is derived from rat pheochromocytoma tumors and has many properties similar to dopaminergic neurons, is one of the most widely used neuronal cell lines for researches on the mechanisms of Parkinson's disease (Jha et al., 2002). In vitro PC12 cell model, we first observed that the neuroprotective effects of baicalein were closely correlated with neurite outgrowth of PC12 cells. The effect of baicalein is very similar to NGF, a well-established inducer of PC12 differentiation

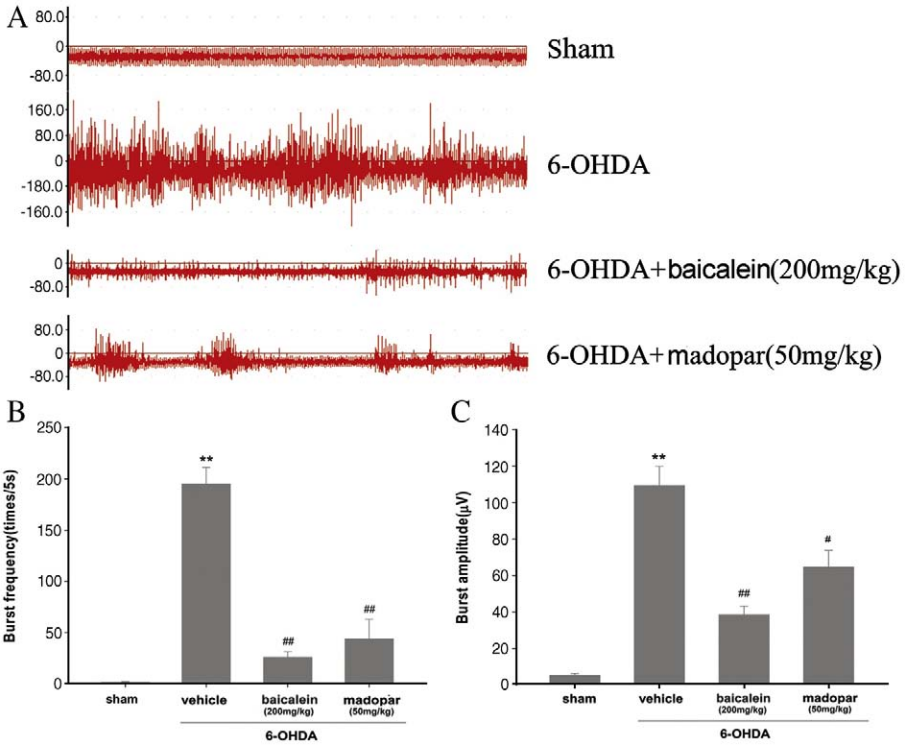

Fig. 5. Effect of baicalein on tremor in 6-OHDA unilaterally lesioned rats. A: Segments of Tremor Monitor activity profiles. B and C: Quantitative determination of burst frequency and burst amplitude by tremor monitor software analysis. Values are expressed as mean ± SEM ($n=12$). $^{*}P<0.05$, $^{**}P<0.01$ compared with sham group, $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with 6-OHDA group.

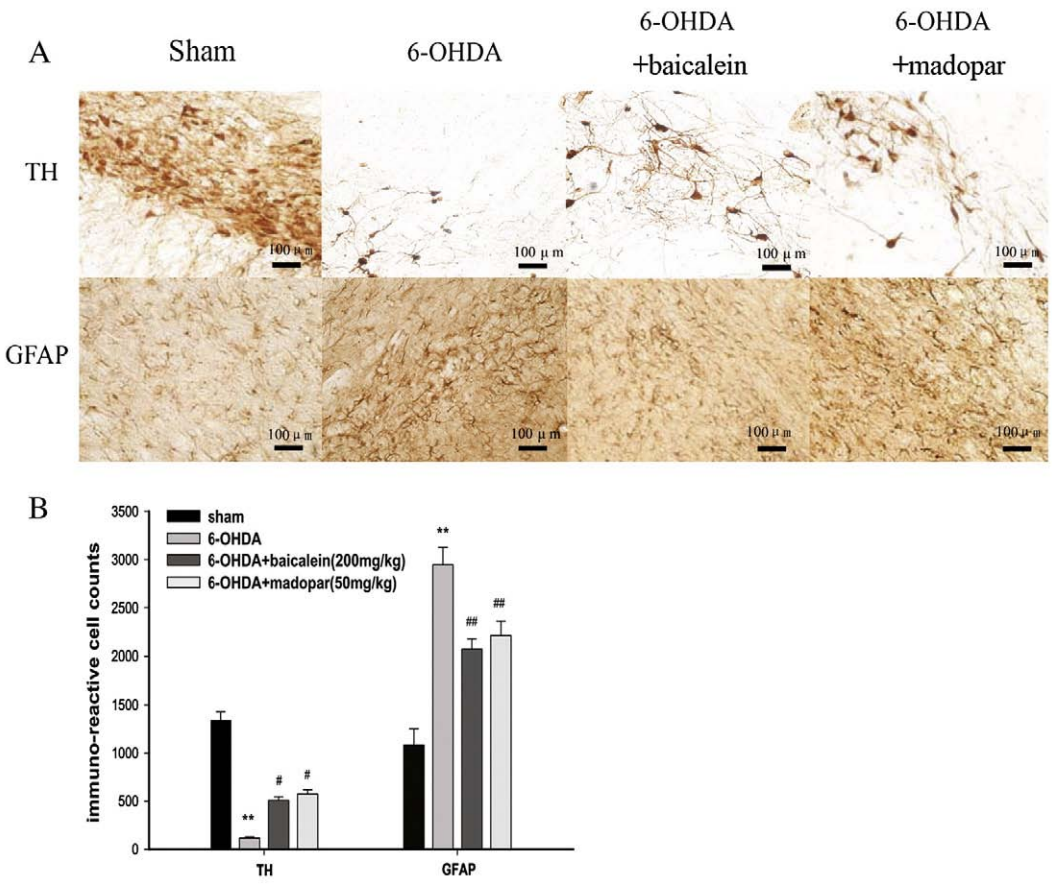

Fig. 6. Effects of baicalein treatment on 6-OHDA-induced changes in tyrosine hydroxylase (TH)-positive neurons and glial fibrillary acidic protein (GFAP)-positive neurons in the substantia nigra. A: Representative microphotographs of TH and GFAP immunostaining in the substantia nigra of rats. B: Summary of the effect of baicalein on 6-OHDA-induced TH and GFAP-immunoreactive neurons in the substantia nigra. The TH and GFAP-positive neurons are expressed as mean ± SEM ($n = 6$). $^{*}P<0.05$, $^{**}P<0.01$ compared with sham group, $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with 6-OHDA group.

(Das et al., 2004; Greene and Tischler, 1976), but its action mechanism needs further study.

It has been generally accepted that rats with a unilateral destruction of the nigrostriatal system by 6-OHDA constituted an animal model for Parkinson's disease (Aguiar et al., 2006; Ungerstedt and Arbuthnott, 1970). It appears to be a valuable model to investigate Parkinson's disease symptomatology and to gain more insight into the possible pathological mechanisms of this neurodegenerative disease (Deumens et al., 2002; Schober, 2004).

In the present study, we have observed an increase in apomorphine-induced rotations in 6-OHDA-lesioned rats. However, baicalein treatment failed to attenuate apomorphine-induced rotations. There may be two reasons responsible for this. On the one hand, injection of 6-OHDA into the MFB unilaterally could cause a total destruction of A9 and A10 cell groups. These lesions were almost complete and few dopaminergic neurons in the SNpc survived. Our treatment may not have such robust protective effects. On the other hand, the rotation does not correlate linearly with the degree of dopamine depletion or restoration (Chang et al., 1999). Experimental manipulation which could reverse rotational behaviors may not necessarily improve motor function (Metz et al., 2001; Metz and Whishaw, 2002). The mechanism of rotation is complicated. In addition, the present study showed that baicalein could significantly decrease the burst frequency and amplitude of muscle activity in 6-OHDA-lesioned rats. Therefore, we conclude that baicalein treatment may have effects on muscle tremor induced by 6-OHDA administration, but have no effects against dyskinesia (e.g. rotational behavior). The mechanism still needs to be explored later.

Moreover, baicalein treatment significantly increased TH-immunopositive neurons in the substantia nigra. These results suggested that baicalein may rescue dopaminergic neurons in 6-OHDA-lesioned animals. The possible mechanism involved in neuroprotective action of baicalein may be its catechol like structure, since it is known that catechol containing compounds are potent radical scavengers and chelators of ferric ion (Mandel and Youdim, 2004). Our results accord with the earlier studies, which found that motor deficits in parkinson's rats have been attenuated by adenosine, selenium and *Ginkgo biloba* (Ahmad et al., 2005; Zafar et al., 2003a,b).

Reactive astrogliosis is a hallmark associated with vigorous response of astrocytes to diverse insults including inflammation (Ridet et al., 1997). GFAP is well known to be a good marker for reactive astrocytes in response to the CNS injury, due to its specificity in astrocytes (Zweig et al., 1992). Recent studies showed that the neuroprotection of baicalein was primarily through the inhibition of inflammatory mediators, ROS and modulation of astrocyte markers associated with response to injury (Sharma et al., 2007). In the present study, GFAP-positive astrocytes were found in the substantia nigra. These observations suggested that an increase in GFAP-immunopositive astrocytes may reflect compensatory action against neuronal cell damage after 6-OHDA lesion. Therefore, it is conceivable that reactive astrocytes may play a key role in the maintenance of injury areas caused by 6-OHDA toxicity. Our findings highlight that baicalein induces neuroprotection through the inhibition of modulation of astrocyte markers.

In conclusion, our results from the in vitro and in vivo studies confirm some protective effect of baicalein in 6-OHDA-induced

cytotoxicity and neurotoxicity. The mechanism is most likely related to its anti-apoptotic, pro-differentiation and anti-inflammatory action. These results suggest that baicalein might be a promising candidate for the prevention or treatment of neurodegenerative diseases such as Parkinson's disease, but further studies to understand the basic mechanism are required.

Acknowledgements

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