Baicalin and deferoxamine alleviate iron accumulation in different brain regions of Parkinson’s disease rats

Pei Xiong, Xin Chen, Chunyan Guo, Nan Zhang, Baocang Ma

Abstract
Previous studies found that iron accumulates in the substantia nigra of Parkinson’s disease patients. However, it is still unclear whether other brain regions have iron accumulation as well. In this experiment, rats with rotenone-induced Parkinson’s disease were treated by gastric perfusion of baicalin or intraperitoneal injection of deferoxamine. Immunohistochemical staining demonstrated that iron accumulated not only in the substantia nigra pars compacta, but also significantly in the striatum globus pallidus, the dentate gyrus granular layer of the hippocampus, the dentate-interpositus and the facial nucleus of the cerebellum. Both baicalin and deferoxamine, which are iron chelating agents, significantly inhibited iron deposition in these brain areas, and substantially reduced the loss of tyrosine hydroxylase-positive cells. These chelators also reduced iron content in the substantia nigra. In addition to the substantia nigra, iron deposition was observed in other brain regions as well. Both baicalin and deferoxamine significantly inhibited iron accumulation in different brain regions, and had a protective effect on dopaminergic neurons.

Key Words
Parkinson’s disease; rotenone; iron; baicalin; deferoxamine; substantia nigra; striatum; hippocampus; cerebellum; neurodegenerative disease; regeneration; neural regeneration

Research Highlights
(1) Iron deposition occurred in the substantia nigra pars compacta, the striatum globus pallidus, the dentate gyrus granular layer of the hippocampus, the dentate-interpositus and the facial nucleus of the cerebellum in a model rat of Parkinson’s disease.
(2) Baicalin and deferoxamine, iron chelating agents, substantially inhibited iron accumulation and had a protective effect on dopaminergic neurons.

Abbreviations
SN, substantia nigra; PD, Parkinson’s disease

INTRODUCTION
In early 1924, researchers found that iron increased noticeably in the brains of Parkinson’s disease (PD) patients, and autopsy results showed that the regions with the greatest iron deposition were the substantia nigra (SN) and the globus pallidus. Imaging studies confirmed iron accumulation in the SN, and the degree of iron deposition was found to be related to the severity of illness. Clinical and animal experiments have shown that iron...
increases significantly in the SN pars compacta of PD patients and animals alike. A recent study detected enhanced echo in the SN using the skull ultrasound method\textsuperscript{[12-14]}, which might be due to iron accumulation and reduced neuromelanin content\textsuperscript{[15]}. Iron deposition also occurs in other chronic central nervous system diseases\textsuperscript{[16]}, such as Alzheimer’s disease and multiple sclerosis. Numerous studies have suggested that the transition element iron is closely related to PD\textsuperscript{[17]}. Abnormal increase of iron in brains might generate free radicals, promote oxidative injury and cause cell death. It is likely one of the factors contributing to neuronal death in neurodegenerative diseases\textsuperscript{[18]}. While this view has become the consensus, the relationship between iron deposition and PD pathogenesis is far from clear. Does iron only accumulate in the SN? Are there any other regions where iron deposition occurs? The answers are unknown. It was previously revealed in our laboratory\textsuperscript{[19]} that there is iron accumulation in the SN of a rat model with a systemic disorder of iron metabolism, with abnormally reduced levels in serum and significantly elevated levels in liver. However, there was no data from our laboratory at the time on whether iron changed in other brain regions. Previous studies found that baicalin, the active ingredient of \textit{Scutellaria baicalensis}, had a protective effect on nigrostriatal dopaminergic neurons in a rotenone-induced rat PD model, as well as in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced mouse model\textsuperscript{[20]}. Baicalin reduced iron deposition in the SN in the rat model\textsuperscript{[20]}. \textit{In vitro}, baicalin inhibited iron accumulation by neural cells and prevented lipid peroxidation due to its ability to chelate iron\textsuperscript{[21]}. This study aimed to examine iron accumulation in different brain areas of a rat model of PD, discuss the relationship between pathological iron deposition and PD, and explore the effects of baicalin and deferoxamine on iron accumulation in different brain regions.

**RESULTS**

**Quantitative analysis of experimental animals**

A total of 84 male Wistar rats were involved in this study. Apart from 11 animals in the normal group (no treatment), the other 73 rats were subjected to rotenone induction to establish the PD model. Thirty-three rats had successful induction and were used in the experiment. They were randomly divided into three groups: model, baicalin and deferoxamine groups. The model group was given saline by gavage. The baicalin and deferoxamine groups were given corresponding intervention for 8 weeks. Four rats in each group were perfused to prepare frozen sections, and three rats were included in data analysis. The remaining seven rats in each group were used for SN iron content determination by inductively coupled plasma mass spectrometer. Finally 44 rats were involved in the final analysis, without any dropout or loss.

**Iron staining in different brain regions of PD rats**

(Figure 1, Table 1)

**Figure 1**  Iron staining in different brain regions of each group.

There were varying degrees of iron accumulation in the striatum globus pallidus (A, F); substantia nigra (B, G); dentate gyrus granular layer of the hippocampus (C, H); dentate-interpositus of the cerebellum (D, I); and facial nucleus of the cerebellum (E, J) in the model group. (F–J, × 200): Enlarged image of the square area in A–E (× 40). Baicalin and deferoxamine significantly inhibited iron deposition in these areas. Scale bars: A–E, 500 μm; F–J, 100 μm.
The positively-stained areas and the integrated absorbance of iron staining in the globus pallidus of the striatum, SN pars compacta, hippocampal dentate gyrus granular layer, dentate-interpositus and facial nucleus of the cerebellum of PD rats were significantly increased compared with the normal control \((P < 0.01)\). The positive areas and integrated absorbance of iron staining in the baicalin and deferoxamine groups treated for 8 weeks were significantly reduced compared with the model group \((P < 0.01; \text{Figure 1, Table 1})\).

**Iron content in the SN of PD rats**

The results of inductively coupled plasma atomic emission spectroscopy showed that iron content in the model group was significantly increased compared with the normal group \((P < 0.05)\). After treatment for 8 weeks, iron content in the mesencephalon of rats in the baicalin and deferoxamine groups was significantly reduced compared with the model group \((P < 0.01 \text{ or } P < 0.05; \text{Figure 2})\).

![Image](image323x332 to 540x434)

**Figure 2** Iron content in the substantia nigra of the rat mesencephalon.

\(^{\text{a}}P < 0.05, \text{vs. normal group}; ^{\text{b}}P < 0.05, ^{\text{c}}P < 0.01, \text{vs. model group}. \text{Data were expressed as mean ± SD} (n = 3), \text{analyzed by one-way analysis of variance and least significant difference t-test.} \)

**Tyrosine hydroxylase expression in the SN of PD rats**

There was a large number of tyrosine hydroxylase-positive cells in the normal group. Cell bodies were uniformly stained with abundant processes. In contrast, there was a remarkable loss of tyrosine hydroxylase-positive cells in the model group. Cell bodies varied in shape, and the number of neurites was evidently reduced. The number of tyrosine hydroxylase-positive cells and the value of the integrated absorbance were significantly decreased compared with the normal group \((P < 0.01)\). After treatment with baicalin or deferoxamine for 8 weeks, the shape of tyrosine hydroxylase-positive cells markedly improved. In addition, the loss of tyrosine hydroxylase-positive cells was reduced. The number of tyrosine hydroxylase-positive cells and the value of the integrated absorbance were significantly increased compared with the model group \((P < 0.01 \text{ or } P < 0.05; \text{Figures 3, 4, Table 2})\).

![Image](normal.png)

**Figure 3** The shape of tyrosine hydroxylase-positive cells in substantia nigra of the rat mesencephalon.

Tyrosine hydroxylase-positive cells were significantly decreased in the model group. After intervention, the loss of cells was noticeably reduced. Rectangle represents region of tyrosine hydroxylase-positive staining. Scale bars: A, 500 μm; B, 100 μm.

![Image](image466x320 to 540x434)

**Figure 4** The number of tyrosine hydroxylase-positive cells in the substantia nigra of the rat mesencephalon.

\(^{\text{a}}P < 0.05, \text{vs. normal group}; ^{\text{b}}P < 0.01, ^{\text{c}}P < 0.05, \text{vs. model group}. \text{Data were expressed as mean ± SD} (n = 3), \text{analyzed by one-way analysis of variance and least significant difference t-test.} \)

| Group     | Globus pallidus of striatum | Substantia nigra | Hippocampus | Dentation-interpositus nucleus of cerebellum | Facial nucleus of cerebellum |
|-----------|-----------------------------|------------------|-------------|---------------------------------------------|-----------------------------|
| Normal    | 798.24±6.16                 | 1300.90±101.07   | 444.82±11.33| 992.70±72.61                                | 23.78±1.62                  |
| Model     | 3069.07±77.90               | 357.24±160.70    | 643.50±5.74 | 2372.90±49.18                               | 1693.60±51.40               |
| Baicalin  | 2230.67±19.20               | 3485.43±50.60    | 593.74±5.30 | 1442.18±15.27                               | 693.54±73.88                |
| Deferoxamine | 2439.20±40.90           | 3710.26±73.60    | 578.30±16.20 | 1496.65±97.24                               | 1343.07±27.00               |

\(^{\text{a}}P < 0.01, \text{vs. normal group}; ^{\text{b}}P < 0.01, ^{\text{c}}P < 0.01, \text{vs. model group}. \text{Data were expressed as mean ± SD} (n = 3), \text{analyzed by one-way analysis of variance and least significant difference t-test.} \)

*Table 1* Effects of baicalin and deferoxamine on integrated absorbance value of iron staining in different brain regions of rats
Behavioral changes in PD rats

Rats in the model group slid down in the inclined plane test more times than normal rats. After 5 and 8 weeks of treatment, the number of times the rats in the baicalin and deferoxamine groups slid down the plane were significantly reduced compared with the model group ($P < 0.01$; Table 3).

| Table 2 | Effects of baicalin and deferoxamine on tyrosine hydroxylase expression |
|---------|-------------------------------------------------------------------------|
| Group   | Integrated absorbance value    |
| Normal  | 200.39±12.83                   |
| Model   | 80.48±4.82*                    |
| Baicalin| 175.12±12.94*                  |
| Deferoxamine | 152.79±13.23*              |

*P < 0.01, vs. normal group; $^*$P < 0.01, vs. model group. Data were expressed as mean ± SD ($n = 3$), analyzed by one-way analysis of variance and least significant difference t-test.

| Table 3 | Effects of baicalin and deferoxamine on the times of slid down in the inclined plane test in a 30-second interval |
|---------|----------------------------------------------------------------------------------------------------------------|
| Group   | Curative time (week) |
| Normal  | 0  | 5  | 8  |
| Model   | 1.82±1.44 | 2.97±1.22 | 2.55±1.22 |
| Baicalin| 5.88±0.69* | 6.18±2.06* | 6.48±2.22* |
| Deferoxamine | 6.88±1.73* | 3.03±1.82* | 3.79±1.92* |

*P < 0.01, vs. normal group; $^*$P < 0.01, vs. model group. Data were expressed as mean ± SD ($n = 11$), analyzed by one-way analysis of variance and least significant difference t-test.

DISCUSSION

Iron is a key factor in neurodegenerative diseases. Disrupted brain iron homeostasis is a characteristic of many degenerative diseases of the nervous system[22]. Abnormal iron deposition in the brain is closely related to PD[14-17, 23]. Iron also accumulates in the SN in the rotenone-induced rat model of PD. Our experiment revealed that iron deposition occurs primarily in five regions; the globus pallidus of the striatum, the dentate gyrus granular layer of the hippocampus, the SN, the dentate-interpositus and the facial nucleus of the cerebellum. Iron accumulated not only in the damaged SN, but in multiple other regions in the rat PD model as well. This excessive iron load might cause degenerative damage to nerve cells in these brain regions.

There was a large quantity of iron deposited in the facial nucleus of the cerebellum in the model group. The facial nucleus is a motor nucleus, mainly related to mastication, swallowing, sucking, expression and pronunciation. It could also affect genioglossus activities[24-25]. PD patients exhibit reduced expression, reduced speech volume, flatness, monotone and dysphagia. The symptoms associated with facial nucleus injury due to iron deposition need to be further studied.

This experiment also found that iron accumulated in the dentate-interpositus nucleus of the cerebellum. Electrophysiological studies have shown that injury or deactivation of the dentate-interpositus nucleus can inhibit the establishment of classical blinking conditioned reflex[26-27]. PD patients exhibit symptoms such as reduced blinking and paesthesia. These symptoms related to nucleus injury due to iron deposition also need to be further studied.

The hippocampus has a crucial role in learning and memory[28]. Does iron deposition in the hippocampal dentate gyrus granular layer cause the injury in this region? Further study is required to determine whether iron accumulation impairs memory function and learning ability and if it underlies affective disorder.

Results showed that iron was abnormally deposited in the striatum-globus pallidus-SN. The loss of nigrostriatal dopaminergic neurons is the fundamental cause of PD. It has been confirmed that posture and gait abnormalities, as well as rigor in the extremities, is directly related to the loss of dopaminergic neurons[29].

Our experimental findings indicate that baicalin and deferoxamine can reduce the loss of tyrosine hydroxylase-positive cells in the SN, improve muscle endurance in model rats and decrease downhill times on the inclined plane. We also found that baicalin and deferoxamine could not only decrease iron deposition in the SN, but in the striatum, hippocampus and cerebellum as well. These results suggest that baicalin and deferoxamine protect dopaminergic neurons by inhibiting iron deposition. Baicalin can pass through the blood-brain barrier[30]. Recently our cytological in vitro experiment showed that baicalin protects cells by chelating iron[21], and we conjectured that it could chelate iron in the brain to inhibit iron accumulation.

Deferoxamine cannot cross the blood-brain barrier. We surmised that deferoxamine inhibits iron deposition in the brain by chelating iron in blood. Baicalin might protect central dopaminergic neurons by a similar mechanism. This suggests that chelating iron in blood could decrease iron deposition in the central nervous system. This opens up new avenues for the development and use of iron chelation in medicine to protect dopaminergic neurons and delay PD neural degeneration.
MATERIALS AND METHODS

Design
A randomized controlled animal experiment.

Time and setting
This experiment was performed at the Pharmacology Laboratory of Traditional Chinese Medicine Academy, Capital Medical University, China from September 2011 to February 2012.

Materials
Animals
A total of 84 male Wistar rats, clean grade, weighing 180–200 g, were provided by the Laboratory Animal Center of Military Academy of Medical Sciences of Chinese PLA, China with certification number of SCXK (Jing) 0011227. The experimental animals were housed at 24°C, in 60% humidity. Animal experimentation was performed in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, promulgated by the Ministry of Science and Technology of China[31].

Drugs
Baicalin powder (99% purity; Nanjing Zelang Medical Technology, Nanjing, China; No. ZL20110708), 3.9 g, was weighed and placed in an amber bottle. 50 mL distilled water was added and sealed airtight. Then, the solution was subjected to 100 Hz ultrasound for 30 minutes to produce a turbid solution (78 mg/mL baicalin). Deferoxamine was provided by Novartis, Beijing, China (No. S1494).

Methods
Establishment of PD model and interventions
Wistar rats were subcutaneously injected with rotenone (2 mg/kg per day; Sigma, St. Louis, MO, USA) for 4–6 weeks, to establish the rat model of PD. Behavioral scores were measured using our neuroethological scoring system[30]. The successful PD model rats were treated for 8 weeks as follows: baicalin group was given baicalin (78 mg/kg per day) by gavage[19, 32]; deferoxamine group was given deferoxamine by intraperitoneal injection (60 mg/kg per day); model group was given an equal volume of saline by gavage (0.1 mL/100 g). All rats were killed after the 8-week intervention. Four rats in each group were fixed in paraformaldehyde and anesthetized with 10% chloral hydrate (0.35 mL/100 g, i.p.), then killed by perfusion with 0.9% saline (400 mL) and 4% paraformaldehyde (350 mL). Brains were removed and stored in paraformaldehyde for 2 hours, then transferred to 30% sucrose solution for dehydration. Frozen sections (50-μm thick) through the striatum, hippocampus, SN and cerebellum were cut on a freezing microtome, and all sections were stained with Perls’ iron staining. Other sections of SN were used for tyrosine hydroxylase immunohistochemistry (mouse anti-rat tyrosine hydroxylase monoclonal antibody; Sigma, 1:2 000). The remaining 7 rats were killed, and the cerebral cortex, striatum, hippocampus, SN and cerebellum were dissected out at low temperature quickly and stored at -80°C for other tests. The SN was used for detection of iron content. Inclined plane test was conducted to assess neurological behavior at 0, 5 and 8 weeks.

Behavioral score system
Based on the neural behavior scoring system established by our laboratory, score 2 or higher was defined as successful establishment of the PD model in rats[19, 20]. Standard for evaluation: Score 1: Resisting arrest behavior was weakened, piloerection, dirty hair, roach back, bradykinesia; Score 2: In addition to score 1 features, initiative activity decline, clear bradykinesia, tremor, instability of gait; Score 4: In addition to score 2 features, instability of gait, or cannot go straight, or unilateral rotation; Score 6: The body inclined towards one side, unilateral forelimb or posterior limb paralysis, walking and feeding difficulty; Score 8: Unilateral forelimb and/or posterior limb complete paralysis, extremity constriction, weight loss, aphagosis; Score 10: Impending death or death. Rotenone injection was stopped as soon as score 2 was successfully reached in the model. Model rats with score 2 were used in this experiment.

Brain iron staining of PD rats
According to a previously described method[33], sections were washed three times for 30 seconds each in distilled water before staining, and then incubated in a freshly prepared solution of equal parts 2% hydrochloric acid and 2% potassium ferrocyanide for 30 minutes at room temperature. After washing with PBS three times, sections were immersed in 99% methanol containing 1% hydrogen peroxide for 20 minutes, washed three times with PBS, and then reacted with 3,3-diaminobenzidine for 30 minutes[32]. Neutral gum was used to seal the sections. Four to six sections were selected from the SN of each rat. Regions of iron staining were visibly stained, being brown and granular. Interspersion, flakiness or clumping was observed in the iron deposits. Regions with significant iron accumulation
were darker, being dark brown or puce. NIS-Elements Br 3.2 pathological image analytical system (Ver.2.1; Tokyo, Japan) and an optical microscope (Nikon, Tokyo, Japan) were used to record intensity-rotated absorbance values.

**Determination of iron content**

SN of rats was digested with 2 mL mixed acid (HNO₃:HClO₄ = 4:1, v/v), adjusted to 3 mL, and iron content was determined with an inductively coupled plasma emission spectrometer (ICTS-7000, Daqjin Company, Japan).

**Determination of tyrosine hydroxylase expression by immunohistochemistry**

Sections were washed three times for 5 minutes each in 0.01 M PBS (pH 7.4), then immersed in 0.3% Triton X-100 for 30 minutes at room temperature. After rinsing in PBS three times, endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 minutes, followed by washing in PBS three times. Specimens were incubated in primary tyrosine hydroxylase antibody (mouse anti-rat tyrosine hydroxylase monoclonal antibody; 1:2 000; Sigma) overnight at 4°C, washed in PBS, then reacted with antibody II 1 (Polink-2 plus Polymer HRP Detection System For Mouse Primary Antibody; Beijing Zhongshan Golden-bridge Biological Technology, Beijing, China; number: K115981K) for 1 hour at room temperature. Sections were then washed with PBS, reacted with antibody II 2 (Polink-2 plus Polymer HRP Detection System For Mouse Primary Antibody; Beijing Zhongshan Golden-bridge Biological Technology; number: K115981K) for 30 minutes at room temperature, washed with PBS, and reacted with 3,3-diaminobenzidine for 30 minutes. Subsequently, sections were sequentially dehydrated in 70%, 80%, 90%, 95% and absolute ethyl alcohol. Sections were then cleared using xylene, and sealed with neutral gum. Four to six sections were selected from the SN of each rat. The number of tyrosine hydroxylase-positive cells was calculated with NIS-Elements Br 3.2 pathological image analytical system coupled with a light microscope (Nikon).

**Assessment of behavioral changes using the inclined plane test**

According to a previously described protocol,[34-35], a large and smooth board was fixed at a 50° angle to the ground. Rats were placed on the top surface of the cardboard, ensuring the head was facing upward. The number of times the rat slid down the inclined plane in a 30-second interval were recorded. This procedure was repeated three times, and the average of the three measurements was recorded for each rat.

**Statistical analysis**

Data were expressed as mean ± SD. Statistical analysis was accomplished using SPSS 13.0 software (SPSS, Chicago, IL, USA). After normality inspection, data were analyzed by one-way analysis of variance and least significant difference t-tests. A P value less than 0.05 was considered to indicate a statistically significant difference.

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**Author contributions:** Pei Xiong was responsible for data acquisition and integration, analyzed experimental data, drafted the paper and provided data support. Xin Chen had full access to study concept and design, validated the paper and was in charge of funds. Chunyan Guo participated in data acquisition and statistical analysis. Nan Zhang was responsible for statistical analysis. Baocang Ma provided data support.

**Conflicts of interest:** None declared.

**Ethical approval:** The experiments were approved by Ethics committee of Capital Medical University in China.

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