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Iron accumulation and microglia activation contribute to substantia nigra hyperechogenicity in the 6-OHDA-induced rat model of Parkinson's disease

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ABSTRACT

Introduction: This study aims to explain the mechanisms for the formation of sonographic features of Parkinson's disease (PD) using a 6-hydroxydopamine (6-OHDA) rat model of PD. The iron chelator deferiprone (DFP) was used in the PD model rat to examine the relationship between iron and the echo signal.

Methods: Rat models were created using stereotactic injections of 6-OHDA. DFP was administered intragastrically. Transcranial sonography (TCS) was performed to observe the substantia nigra (SN) echo signal of the brain. Immunofluorescence and iron staining were performed to observe the histological characteristics of the hyperechogenic area. The imaging findings were compared with the histopathological findings.

Results: The PD model rat presented a large area of hyperechogenicity in the SN. Ferric ion accumulation and microglia proliferation occurred in the hyperechogenic area. DFP inhibited dopaminergic (DA) neuron necrosis, ferric ion accumulation and microglia proliferation and reduced the hyperechogenic area of the SN.

Conclusions: Both iron aggregation and gliosis contribute to the formation of substantia nigra hyperechogenicity (SNH) in PD. DFP exhibits a neuroprotective effect by inhibiting SNH. Iron deposit and the SNH are correlated with DA neuron necrosis.

1. Introduction

Parkinson's disease (PD) is a severe neurodegenerative disease that mainly affects elderly individuals. The existing diagnostic criteria for PD mainly rely on the emergence of motor symptoms and a series of clinical measurements [1]. As more than half of nigral dopaminergic (DA) neurons have already degenerated upon the emergence of the initial movement symptoms of PD, the current methods of diagnosis are typically effective only in patients with advanced-stage PD. An accurate and practical method for the early diagnosis of PD is needed to identify early-stage PD patients who lack severe symptoms [2]. To administer effective neuroprotective treatment, it is essential to find biomarkers for the early diagnosis of PD.

Since the first description of substantia nigra hyperechogenicity (SNH) in transcranial sonography (TCS) of PD patients by G. Becker et al. [3], an increasing number of clinical studies have been performed about SNH in PD patients [4]. To date, SNH presented in TCS has proven to be useful for the early diagnosis of PD [5]. However, the underlying mechanism contributing to SNH is unclear. Iron plays an important role in neurodegeneration process [6]. Several studies have demonstrated that a correlation exists between SNH and tissue iron content [7,8]. To our knowledge, there is no study on the relationship between iron and SNH in PD rat model. Whether iron deposit is the only cause of SNH is not yet conclusive.

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To examine the relevance of SNH to iron deposit, we established a 6-hydroxydopamine (6-OHDA) stereotactically injected PD rat model and determined whether SNH was present in this vivo model. We furthermore used the iron chelator deferiprone (DFP) to inhibit the transport of iron and observed the ultrasound changes in the substantia nigra (SN) using pathological and molecular biological methods.

The aim of our study was to examine the relationship between iron and SNH and to explain the mechanism of formation of the sonographic features of PD.

2. Materials and methods

2.1. Chemicals and antibodies

The chemicals used included 6-OHDA (Sigma-Aldrich, St. Louis, MO, USA), DFP (Sigma-Aldrich, St. Louis, MO, USA) and 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA). A ferric ion staining kit (Senbeijia Biotech Co., Nanjing, China) was used to detect ferric ion deposit. The antibodies used for immunofluorescence and Western blotting consisted of anti-tyrosine hydroxylase (TH) (Sigma, St Louis, MO, USA), anti-ionized calcium binding adaptor molecule 1 (Iba-1) (Wako-chem, Japan).

2.2. Animals and tissue preparations

Twenty-seven male Sprague-Dawley rats (250–300 g, 12 weeks old) were purchased from the Experimental Animal Center of the Fourth Military Medical University. The rats were used according to the Guidelines for Animal Care and Use of the Fourth Military Medical University. All efforts were made to minimize the suffering of animals throughout the study process. The animals (three per cage) were permitted free access to food and water under a 12:12 h light/dark cycle.

Animals were randomly divided into three groups: a sham group (n = 9), a 6-OHDA lesion group (n = 9), and a 6-OHDA + DFP (150 mg/kg/day) group (n = 9). The DFP oral solution was prepared freshly before each gavage. DFP (dissolved in 2 mL saline) was administered by intragastric infusion twice daily for 24 consecutive days starting from 2 days before the 6-OHDA lesion was generated. On day 24, TCS was performed. After TCS, the rats received intracardiac perfusion with saline and 4% paraformaldehyde. The brains were then dehydrated in a series of 20% and 30% sucrose solutions before dissection. Coronal sections (20 μm thickness) encompassing the SN were collected and then used for staining.

2.3. 6-OHDA lesion

Rats were anesthetized with intraperitoneal infusions of chloral hydrate (350–400 mg/kg) and fixed on a stereotaxic frame. Two burr holes were drilled into the skull above the region of the right SN according to the following coordinates: (1) anterior-posterior (AP): −5.2 mm; medial-lateral (ML): −1.9 mm; deep ventral (DV): −8.2 mm, (2) AP: −5.5 mm; ML: −2.4 mm; DV: −7.6 mm. A total volume of 6 μL of 6-OHDA (2.5 μg/μL) was microinjected into the right SN at a rate of 1.0 μL per min. The microinjection needle was left in place for 5 min following injection before it was slowly extracted. Rats in the sham group underwent the same surgical procedure and were injected with an equal volume of saline. Rats were placed in clean cages on warming pads for recovery after surgery.

2.4. Ultrasound examination

The rats were anesthetized intraperitoneal with chloral hydrate (350–400 mg/kg). The skull of the rat then was carefully removed before ultrasound examination for better observation. The probe was held above the brain, and warm coupling gel was placed into the gap between the probe and brain tissue to isolate it from the air.

Ultrasound examination was performed following a standard protocol. A sonographic device (LOGIQ E9, General Electric Company, England) with a 15-MHz phase array transducer was used. The sonographer was experienced in animal ultrasound protocols. The parameter settings were fixed throughout the entire study at a depth of 3 cm and a dynamic range of 35–55 dB.

2.5. Immunofluorescence

Brain sections were incubated with 0.1% Triton X-100 for 30 min followed by three washes with phosphate buffer solution (PBS) and then blocked with 5% goat serum in PBS for 30 min. Afterward, sections were incubated with primary antibodies overnight at 4 °C followed by three washes with PBS, then incubated with a fluorescence-conjugated secondary antibody for 2 h at room temperature and washed three times with PBS. The nuclei were counterstained with DAPI. Fluorescence images were obtained using a confocal laser microscope (Nikon, C2 Si, Tokyo, Japan).

2.6. Iron staining

Iron staining was used to detect ferric ion deposit. Brain sections were rinsed with distilled water and then immersed in a mixture of equal volumes of 1% HCl and 1% potassium ferrocyanide (v/v) for 30 min. The sections were then re-rinsed with distilled water and stained with nuclear fast red dye for 10 min.

2.7. Western blotting

The tissues were lysed in lysis buffer (10 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, 1 mM EDTA, pH 7.4) and mixed adequately with protease and phosphatase inhibitor cocktails (Roche). The protein samples were prepared by boiling at 95 °C for 15 min, resolved by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% bovine serum albumin at room temperature for 2 h and then incubated with the indicated primary antibodies by gentle shaking overnight at 4 °C. After three washes with TBST, the membrane was incubated with HRP-conjugated secondary antibodies. Protein bands were detected using ECL, and the bands densities were quantified with ImageJ software (NIH, Bethesda, MD).

2.8. Statistical analysis

Data from at least three independent experiments were analyzed by one-way ANOVA. All results are expressed as the means ± standard error of the mean (SEM). A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. 6-OHDA induced SNH, whereas DFP attenuated SNH

A large area of hyperechogenicity in the SN and adjacent areas was evident on the side with the 6-OHDA lesion in the rats of the 6-OHDA group rats (Fig. 1A). Only the hyperechoic line of the needle pathway was found in the sham group rats (Fig. 1A). Patch or punctiform hyperechogenicity was observed in the 6-OHDA + DFP

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