Evaluation of Parkinson’s disease in 3 rat models induced by three different unilateral injections of 6-hydroxydopamine

ABSTRACT
This study was designed to investigate the difference between three 6-hydroxydopamine (6-OHDA)-induced rat PD models. Those rats model were established by stereotoxic unilateral 6-OHDA injection into different parts of nigrostriatal pathway including the striatum (ST), the substantia nigra pars compacta (SNpc) and the ventral tegmental area (VTA) in left side of rat brain. To detect pathological change in the unilaterally lesion 6-OHDA rats, the intact hemisphere work as a internal control. Immunohistochemical staining was used to evaluate the expression of tyrosine hydroxylase (TH) in substantia nigra pars compacta, as indication of injure of dopaminergic neuron. Remarkable TH-positive neuron loss was found in substantia nigra pars compacta in all 6-OHDA injected groups. The steep depletion of the content of dopamine and its metabolites of in striatum was also observed in all 6-OHDA injected groups. The 6-OHDA-impaired rats demonstrate a decrease of body weight compared to sham group rats. Behavioral assessments of motor impairments in the unilateral 6-OHDA rat model were done by apomorphine-induced rotation tests. In apomorphine-induced contralateral rotations test, the ST injection group show increasing rotation from the 2th week to the 4th week and high success rate of modeling; SNpc group and VTA + SNpc groups showed stable rotations from the 2nd week but low success rate. These results suggested that ST group has higher success rate and is more practicable than the other two groups.

KEYWORDS 6-hydroxydopamine; rat model, Parkinson’s disease; striatum; substantia nigra

INTRODUCTION
Parkinson’s disease (PD) is the second popular neurodegenerative disorder to which the elderly are vulnerable. PD is primarily defined by progressive degeneration of dopaminergic neurons in the SNpc, subsequently resulting in decrease of dopamine which are consistent with motor impairment of PD patient1-3. Currently, as there is no therapy which can completely halt or reverse the progress of PD, the more promising treatments for this devastating disease are urgently needed. To achieve this arduous goal, a broad variety of PD model have been developed to explore the unknown etiology of PD and the underlying mechanisms of neurodegeneration4. A perfect model of PD which should reproduce pathological characters and clinical features similar to human Parkinson’s disease should be considered to be first priority to study PD. Despite the fact that none of the current PD models meets the requirement, animal models have contributed significantly to our current deep understanding of the disease process and potential therapeutic targets in PD5.

The application of toxin-induced model has play extremely important role to unveil the pathophysiology of underlying PD6. The most frequently used neurotoxin in rodent PD models is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-OHDA and rotenone, etc. 6-OHDA, a representative hydroxylated analog of dopamine, selectively destroys catecholaminergic neurons in sympathetic adrenergic nerve teriminals7-9. Metabolism of 6-OHDA leads to numerous phenomenon of oxidative stress, and the formation...
of free radicals consequently results in damage of dopamine neurons antioxidant system, mitochondrial function, membrane stability and the integrity of the DNA to cause cell death, which eventually leads to nigrostriatal dopamine dysfunction and Parkinson-like motor impairment.

Unilateral 6-OHDA-lesioned rat PD model is the most widely used model because of pathological characteristics similar to that of PD patients. Currently, there exist various methods to manipulate 6-OHDA induced rat PD models. Diverse injection site and injection dose can lead to difference of PD models. Furthermore there are still some limitations of PD models reported in literature, such as low success rate, high mortality.

More efforts should be taken to build up a more suitable model. Unilaterally injecting 6-OHDA is more preferential than bilaterally injecting. Hence, in this experimental study unilaterally stereotactic injection of 6-OHDA is used to establish three different rat models including two sites that are located in same point but different depth within ST, single site within SNc, double site concerning SNc and VTA. Apomorphine-induced rotations test, quantitative analysis of dopamine in striatum by HPLC-ECD and qualitative analysis of TH-positive neuron by immunohistochemical staining are conducted to assess the performance of different methods. Through a comprehensive comparison of the three models, we hope to find out a reliable method to manipulate PD model with high success rate for further exploring.

MATERIALS AND METHODS

Chemicals and reagents

6-hydroxydopamine (6-OHDA, Sigma Chemical Co. St. Louis, MO, USA), was made freshly on the days of experiment by dissolving at a concentration of 5 μg/ml saline in 0.1% ascorbic acid. Apomorphine (Sigma St. Louis, MO) was injected i.p. in the rat at a dose of 0.5 mg/kg 10 min before the start of the Rotometer test. Rabbit anti rat anti-TH monoclonal antibody (Millipore) and GTVisionTM III Detection System/Mo&Rb (Gene Tech) were purchased from Sigma-Aldrich. All other chemicals and regents were of the highest grade available from Sigma-Aldrich unless stated otherwise.

Animal

Male SD rats (3 months old, 200–250 g) were purchased from Animal Center of Guangdong Province. Animals were kept under the same environmental conditions (ambient temperature 23–25°C, humidity 50–65%, 12 h light/dark cycle) and received food and water ad libitum. Animals were raised for 1 week in the conditions described above before 6-OHDA injection. All animal studies were conducted according to guidelines of the experimental animal care and use committee of Jinan University. All procedures in this study were in agreement with the Guide of Care and Use of Laboratory Animals from Jinan University and the National Institutes of Health guide.

TREATMENT

Animals were randomly assigned to four groups (n = 10 for each group): sham group (4 μL of 0.1% ascorbic acid in 0.9% NaCl); ST group (4 μL of 5 μg/mL 6-OHDA in 0.1% ascorbic acid and injected at the same injection site with two injection depth); SNpc group (4 μL of 5 μg/mL 6-OHDA in 0.1% ascorbic acid); VTA + SNpc group (2 μL of 5 μg/mL 6-OHDA in 0.1% ascorbic acid at each injection site). Rats were anesthetised with isoflurane and placed on a stereotaxic frame with non-traumatic ear bars (RWD LIFE SCIENCE, China). The scalp of rat was cut and retracted to expose the skull. Holes were drilled above the right injection site. Drugs were unilaterally injected into the targeting site by a 28-gauge needle Hamilton syringe (Hamilton Company, Switzerland). The injection rate was 1 μL/min and the cannula was indwelled for 10 min after the injection at a rate of 1 mm/min. After the surgery, all animals were intramuscular injected penicillin (0.2 mL of 50 × 10⁴ units in 0.9% NaCl) for 5 days. The coordinates were established according to the SD rat Stereotactic Brain Atlas. Coordinates were as follows:

- Sham group and ST group directly into two sides of the left striatum, coordinates related to bregma, AP = 0 mm; ML = −3.0 mm; DV = −5.5 and −4.5 mm; The SNpc group was injected at the site: coordinates related to bregma, AP = −5.2 mm; ML = −1.8 mm; DV = −7.8 mm;
- The SNpc + VTA group was injected two sites: the coordinates related to bregma, AP = −5.2 mm; ML = −1.8 mm; DV = −7.8 mm and the coordinates related to bregma, AP = −4.6 mm; ML = −0.9 mm; DV = −7.8 mm.

ROTATIONAL BEHAVIOR

The behavioural experiment was performed by an observer who is blinded to the experiment. At the 2nd, 3rd and 4th week after surgery, Apomorphine-induced rotation test was conducted. Rats were placed in individual glass bowls with a diameter of 40 cm and adapted for 10 min before injection; 10 min after intraperitoneally (ip) apomorphine administration, the rotations of rats were recorded for total 30 min and the total number

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<th>Table 1</th>
<th>The injection site of three rat PD models.</th>
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<td>AP (mm)</td>
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<td>ST</td>
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<td>0</td>
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<tr>
<td>SNpc</td>
<td>−5.2</td>
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<td>SNpc + VTA</td>
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of contralateral rotation was counted. Only rats whose rotations were more than 7 rpm/min are considered to be successful PD models.

**MEASUREMENT OF DOPAMINE (DA) DIHYDROXY PHENYL ACETIC ACID (DOPAC) AND HOMOVANILLIC ACID (HVA) CONTENTS IN STRIATUM OF RATS BY HPLC ASSAY**

Rats (n = 5 in each group) were decapitated and the brains were immediately taken out and rinsed in ice-cold isotonic physiologic saline for biochemistry evaluation after three weeks of behavioural test. Lesioned (left) striatum and unlesioned (right) striatum tissues were separately isolated and kept frozen in liquid nitrogen before analysis. The right striatum tissues from every group were used for internal control in all the experiments of the present study. ST tissues were weighted and processed with 0.8 M perchloric acid (V/M =10:1) for removing the impurities, then centrifuged at 4°C for 15 min at a rate of 12,000 g. The supernatant solutions were collected and measured the concentrations of DA, DOPAC and HVA by high-performance liquid chromatography (HPLC) with the electrochemical (EC) assay (Waters 2465, USA). We set the rate at 1 ml/min, column temperature at 33°C and detection voltage 0.52 V. The PH value of mobile phase was adjusted to 3.5.

The content of DA, DOPAC and HVA were calculated with the standard curve by peak area. The units of DA, DOPAC and HVA were quantified by representation of ng per mg of tissue.

The standard curves are as below:
- DA: \[ Y = 7.6682X + 0.9942 \quad (R^2 = 0.9999) \]
- DOPAC: \[ Y = 7.5563X + 1.8354 \quad (R^2 = 1) \]
- HVA: \[ Y = 1.4834X + 14.574 \quad (R^2 = 0.9998) \]

**IMMUNOISTOCHEMICAL ASSAY**

To detect the damage of neuron in substantia nigra, rats which have been modeled successfully in three groups were randomly anesthetised with 10% chloral hydrate (0.35 ml/100 g, i.p.) and transcardially perfused with saline and 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Brains were collected and then post-fixed in 4% PFA overnight at 4°C. After that, they were washed with PBS, and gradually dehydrated in 10%, 20% and 30% gradient sucrose solution at 4°C for at least 24 h until the tissues sank to the bottom. Coronal sections (30 μm, from bregma –4.0 mm to bregma –6.0 mm) were obtained with a Leica freezing microtome. Sections were processed for TH immunohistochemistry as the method described below. The slides were incubated with a horseradish peroxidase conjugated secondary antibody (goat anti-rabbit IgG, 1:500) for 1 h at room temperature. The colour was developed using DAB as a chromogen. TH-positive neurons in SNC were observed by OLYMPUS X701.

**STATISTICAL ANALYSIS**

Each data point represents grouped data from 10 animals. One-way ANOVA with Tukey’s test and two-way ANOVA followed by the Newman–Keuls test were used to determine significance. Data are expressed as mean ± standard errors and deemed significant when P < 0.05.

**RESULTS AND DISCUSSION**

**Effect on body weight of three methods**

Experimental data shows that 6-OHDA-impaired rats demonstrate a decrease of body weight compared with sham group rats. As shown in Fig. 1, the 6-OHDA administered ST group rats showed fewer increment of body weight than the other group rats (P < 0.001).

**Apomorphine-induced Contralateral Rotations**

After 2, 3, 4 weeks of surgery, the apomorphine was administrated intraperitoneally and the apomorphine-induced contralateral rotations in each group were counted. Results were expressed as contralateral net turns/min. As shown in Fig. 2, since the 2nd week the average rotational numbers of VTA + SNpc double point injection groups and SNpc single point injection group remain invariable which indicates the injure caused by 6-OHDA have already retained. However, the success rate of ST group was unstable until the 4th week, which means a prolonged lesion towards nigrostriatal pathway. ST group-administered rats exhibited more rotational numbers and higher success rate than the other groups at the 4th week.

**REFERENCES**


![Fig. 1](image-url) Data represent the body weight (mean ± S.E.M.) of control animals (N = 10) and rats that 6-OHDA administered group. Two-way ANOVA followed by the Newman–Keuls test showed significant differences between striatum compared to sham group (P < 0.001).
Three weeks after 6-OHDA injection, the success rate approached approximately to 90% in ST group rats (Fig. 3).

NEUROCHEMICAL DETREMINATIONS

As shown in Fig 4, unilateral injection of 6-OHDA led a unilateral lesion of dopaminergic cell in substantia nigra. In turn it give rise to axonal loss in striatum. In ST group, VTA + SNpc group and SNpc group, the content of DA and its metabolites of the lesioned side were severely reduced compared with that of the unlesioned side. The SNpc group showed at least 90% decrease in DA and its metabolites contents in the lesioned side relative to the corresponding unlesioned side. The severe reduction of DA which also is reported in PD patients to some extent marks the success of model.

TH-POSITIVE CELLS IN SUBSTANIA NIGRA IN 6-OHDA-TREATED RAT

Expressions of TH protein can represent the amount of dopaminergic neurons. As shown in Fig. 5, it illustrates remarkable reduction of the number of TH-positive cells in substantia nigra of 6-OHDA-treated animals. The loss of dopaminergic neurons of the lesioned side (left) in the midbrain of all three model rats is obvious compared to the unlesioned side (right), which indicates the destructive damage caused by 6-OHDA.

DISCUSSION

PD is characterized by a loss of dopaminergic neurons or Lewy body found in the survived dopaminergic neurons, especially in the SNpc, resulting in depletion of striatal dopamine, a neurotransmitter regulating excitatory and inhibitory impulses from basal ganglia. Therefore, the investigation of PD extremely rely on the effective experimental animal model bearing pathological similarity with PD patients. 6-OHDA toxicity through generating reactive oxygen species selectively destroys dopamine neurons. Increasing studies have also certified...
that 6-OHDA-mediated neurotoxic pathological manifestations in rodent model have successfully mimicked clinical symptoms of PD patients.\(^2\)

As 6-OHDA cannot cross the blood brain barrier, 6-OHDA has to be administrated stereotactically into brain. In this study we investigated the performance of three different PD models induced by brain stereotactically injection into the nigrostriatal pathway. One or two point injections are in different sites involving SNC, the ST and midbrain VTA.\(^2\)

The devastating damage in dopaminergic neuron and dramatic reduction of dopamine in striatum has been detected in all three 6-OHDA unilaterally injected rat models.

Precisely positioning which largely determine the success rate of modeling is extremely difficult, in particular, for the small substantia nigra part. The reported success rate of 6-OHDA injection into SNpc is close to 30%. The success rate of VTA and SNpc double point injection method is appropriately to 40–50%. However, mechanical damage during the surgery is too severe to recover for rats. As striatum is much larger than both SNpc and VTA, intrastriatal injection of 6-OHDA can achieve the highest success rate but lowest fatality rate. Above all, application of the unilateral striatum injection (two injection depth at the same injection site) is able to attain more than 90% success rate.

The apomorphine-induced rotational behaviour can predict the extent of cell dopaminergic neuron loss. Thus, the gradually increasing rotations of intrastriatal injection group means the progressive lesion in substantia nigra which resembles the increasing sever symptom of PD patient.\(^8\) On the other hand, the rats that are injected with 6-OHDA in both SNpc and VTA show stable rotations since 2 weeks after surgery. It probably suggests the rapidity of neuron death induced by 6-OHDA.

**CONCLUSION**

Regardless of difference of success rate and the length of time of model reaching stable, this study demonstrates that all the three modeling methods are able to replicate those critical pathological feature of PD patient, such as severe loss of dopaminergic neuron and subsequent depletion of dopamine in striatum. Furthermore, this study also confirmed that 6-OHDA injection into the striatum can be simple and convenient for preparing PD rat model. It is not only easier to manipulate, but also share more similarity to the clinical symptom of PD patients than two other models for it causes a more progressive, retrograde-induced neuron death.\(^2\)

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**REFERENCES**

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