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Gastrointestinal Non-motor Dysfunction in Parkinson's Disease Rats with 6-hydroxydopamine

Xiao-Yan Feng*, Jing-Ting Yan, Xiao-li Zhang and Jin-xia Zhu

Department of Physiology and Pathophysiology, School of Basic Medical Science, Capital Medical University, Beijing, China

*Corresponding authors:

Xiaoyan Feng, PhD

Department of Physiology and Pathophysiology

School of Basic Medical Science

Capital Medical University

No. 10 Xitoutiao, You An Men, Beijing 100069, China

E-mail: fengxy@ccmu.edu.cn

Tel: (86)10-8391-1832

Short Title: Gastrointestinal dysfunction in the 6-OHDA rats

Abbreviations: PD=Parkinson's disease; DA=dopamine; GI=gastrointestinal; 6-OHDA=6-hydroxydopamine; K-HS=Krebs-Hensleit solution; I_{SC} = short-circuit current; UPLC-MS/MS=ultra-performance liquid chromatography tandem mass spectrometry; PBST=phosphate buffer solution; TER=transepithelial resistance.

Summary

Parkinson's disease (PD) is a neurodegenerative disease with a progressive loss of mesencephalic dopaminergic neurons of the substantia nigra (SN). To further evaluate its pathophysiology, accurate animal models are needed. The current study aims to verify the impact of a 6-hydroxydopamine (6-OHDA) bilateral microinjection into the SN on gastrointestinal symptoms in rats and confirm that the 6-OHDA rat model is an appropriate tool to investigate the mechanisms of Parkinsonian GI disorders. Immunohistochemistry, digital X-ray imaging, short-circuit current, FITC-dextran permeability and ultra-performance liquid chromatography tandem mass spectrometry were used in this study. The results indicated that the dopaminergic neurons in SN and fibres in the striatum were markedly reduced in 6-OHDA rats. The 6-OHDA rats manifested reductions in occupancy in a rotarod test and increases in daily food debris but no difference in body mass or daily consumption. Compared with control rats, faecal pellets and their contents were significantly decreased, whereas gastric emptying and intestinal transport were delayed in 6-OHDA rats. The increased *in vivo* FITC-dextran permeability and decreased intestinal transepithelial resistance in the model suggest attenuated barrier function in the digestive tract in the PD model. Moreover, inflammatory factors in the plasma showed that pro-inflammatory factors IL-1β and IL-8 were significantly increased in 6-OHDA rats. Collectively, these findings indicate that the model is an interesting experimental tool to investigate the mechanisms involved in the progression of gastrointestinal dysfunction in PD.

Keywords: Parkinson's disease, gastrointestinal dysfunction, 6-hydroxydopamine

INTRODUCTION

Parkinson's disease (PD) is a chronic, progressive dopaminergic neurological disorder, which is often accompanied by motor dysfunctions, such as resting tremor and rigidity, and various non-motor symptoms, especially gastrointestinal (GI) dysfunctions including gastroparesis, constipation and duodenal ulcer (Odin *et al.*, 2018; Sauerbier *et al.*, 2017; Shen *et al.*, 2017). It has been reported that motor symptoms are realized after a loss of more than 70% of the dopaminergic neurons in the substantia nigra (SN) (Ferro *et al.*, 2005), but a modest reduction in dopamine content is sufficient to cause GI dysfunction before the occurrence of motor disorders (Zheng *et al.*, 2014). Clinical research also suggests that GI dysfunctions frequently appear in the early stages of the disease or even many years before motor impairment.

Animal models have been acknowledged as useful and important tools to analyse the pathogenic mechanisms of manifestations and potential therapeutic agents in PD (Grandi *et al.*, 2018; Jakaria *et al.*, 2018). Neurotoxic agents include rotenone, 6-hydroxydopamin (6-OHDA), lipopolysaccharide (LPS) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Johnson *et al.*, 2015; Marin *et al.*, 2015) and dopaminergic neurotransmission drugs, such as reserpine (Shireen *et al.*, 2014) or genetic manipulation (Imbriani *et al.*, 2018), have been administered to animals to mimic the characteristic symptoms of PD. In all of the above cases, unilateral administration of 6-OHDA to the medial forebrain bundle (MFB) (Boix *et al.*, 2015) or the SN (Kim *et al.*, 2016) was the most widely used PD models. However, PD affects both brain hemispheres, and GI functions are bilaterally controlled. Compared

to the unilateral 6-OHDA rat model, bilateral models more closely approximate the real pathological situation and exclude compensation for the lesion side by the intact site (Deumens *et al.*, 2002). In the present study, we aimed to verify the impact of bilateral microinjection of 6-OHDA into the SN on gastrointestinal symptoms in rats and confirm the 6-OHDA rat is an appropriate experimental tool to investigate the mechanisms involved in the progression of GI dysfunction in PD. Metabolic measurement, digital X-ray imaging, short-circuit current and FITC-dextran permeability were used to determine the reproducibility of the bilateral 6-OHDA model and its ability to the mimic the many physiological features of PD.

METHODS

Drugs and solutions

Dopamine hydrochloride, 6-hydroxydopamine hydrochloride, FITC-dextran (Sigma-Aldrich, St. Louis, MO, USA), and barium meal (Kangte Biological Engineering Co., Ltd, Jiangsu, China) were used in the present study. The Krebs-Henseleit solution (K-HS) contained the following (in mmol/l): NaCl 117, KCl 4.7, $MgCl_2$ $6H_2O$ 1.2, $CaCl_2$ $2H_2O$ 2.5, $NaHCO_3$ 24.8, KH_2PO_4 1.2 and glucose 11.1. The solution was gassed with 95% O_2 and 5% CO_2, and HCl was used to adjust the pH to 7.4.

Animals and tissue preparation

Animal Care

All male Sprague-Dawley rats (210-230 g) were purchased and maintained in the animal facilities at the Laboratory Animal Services Center of Capital Medical

University. The animals were housed in a light-dark cycle of 12:12 h and provided free access to food and water. All of the experiments were performed in accordance with the guidelines established by the Beijing Administration Office of Laboratory Animals and following the Administration Regulations on Laboratory Animals of Beijing Municipality.

6-OHDA Rats

The methods for producing 6-OHDA rats have been previously described (Feng *et al.*, 2017). Briefly, the rats were received bilateral infusions of 6-OHDA (4 μg in 2 μl of 0.9% saline containing 0.05% ascorbic acid for each injection site) into the SN using a 10 μl Hamilton syringe. The control rats received 0.2% ascorbic acid/saline.

At 4 weeks after 6-OHDA treatment, each rat was transferred into an individual metabolic cage (Ugo Basile, Gemonio VA, Italy) and observed throughout a 24 h period to monitor the daily food and water consumption for one week. The food residue and stool samples were collected and measured every day during the fifth week. The solid matter of the stool was dried in an oven at 60℃ for 12 h.

Tissue Preparation

The rats were killed by decapitation at the sixth week. The brains were immediately removed and immersed in 4% paraformaldehyde (12 h) for post-fixation and then placed in 30% sucrose (48 h) for dehydration. The brains were retained for immunohistochemistry. Then, the abdominal wall was opened. The duodenum next to the gastric antrum and the distal colonic segment away from the anus (approximately 2 cm) was quickly removed and immersed in K-HS. Each segment (1 cm) was cut longitudinally along the mesenteric border and cleaned. The duodenal/colonic tissue

was pinned (mucosal side down) in a Sylgard-lined Petri dish to strip away the serosa, muscularis and submucosa with fine forceps. The duodenal/colonic mucosa preparations were obtained for *in vitro* short circuit current measurement.

Immunohistochemistry

The brain slices were fixed with cold acetone for 15 min, and then washed (3×5 min) in 0.3% Triton X-100 phosphate buffer solution (PBST) to eliminate the residual fixative. After blocking with 3% H_2O_2 and 10% goat serum (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 30 min, the sections were incubated with TH antibody (Mouse, 1:10000, Sigma/T1299) at 4 ℃ overnight. After washing in PBST (3×5 min), sections of the SN were incubated with donkey anti-mouse IgG (1:1000, Invitrogen/A21203) for 1 h at room temperature and then observed under a fluorescence microscope (Leica DM LB2, St. Gallen, Switzerland). The sections of the striatum were incubated with sheep anti-mouse IgG (1:1000, Rockland/13175) for 1 h at room temperature and incubated with 3, 3'-diaminobenzidine tetrahydrochloride (DAB Substrate Kit for Peroxidase, Beyotime Biotechnology, Shanghai, China) for 2 min, stopped with water, and then placed in xylene and overlaid with a coverslip using neutral resin-mounting medium.

Ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS)

The SN, striatum and DMV tissues were harvested from the rats at the sixth week. The DA content in these tissues was measured by UPLC-MS/MS analysis, which has been described elsewhere (Zhang *et al.*, 2015). Briefly, each sample was weighed and

homogenized in 2% aqueous formic acid. The homogenates were ultrasonically dissociated with a mixture of acetonitrile/methanol/formic acid and centrifuged. The supernatant was evaporated to dryness and re-dissolved with reconstitution solvent followed by another round of centrifugation. The supernatant was immediately used for UPLC-MS/MS analysis (Key Laboratory of Radiopharmaceuticals, Ministry of Education, College of Chemistry, Beijing Normal University).

Rotarod test

A rotarod test was used to evaluate motor coordination by measuring the ability of a rat to stay on a rotating drum. At the fifth week after 6-OHDA treatment, the rats were placed on the rotarod instrument (diameter 3.75 inches, Acceler Rotarod; Jones & Roberts Company, Olympia) at a fixed speed for adaptive training before testing (3-5 min, 3 times each day for 3 consecutive days). On the test day, the rats were trained for 3-5 min until they adapted to 8 rpm/min on the rotarod. After the rats were balanced, the drum was gradually accelerated until the rats fell off of the drum. The time and speed to fall was recorded by a sensing platform. Each rat was given 3 trials, and the mean time of the 3 trials was calculated.

Gastrointestinal motility

Gastric emptying was assessed using an *in vivo* digital X-ray imaging. Each animal received 3 mL of a barium meal (barium sulphate) through oral gavage after fasting for 20 h. The Kodak In Vivo Imaging System FX was used to obtain the gut plain radiographs with a manual focus distance of 50±1 cm and an exposure time of 30 s. The images were recorded every 45 min after barium meal ingestion. The barium sulphate content in the stomach was measured with area and greyscale. The intestinal motility was measured by the total intestinal transit time, which was recorded by the

158 first stool including barium sulphate.

159 **Short-circuit current (I_{SC}) measurement**

160 The duodenal/colonic mucosa preparations were mounted between the two halves of
161 an Ussing chamber, bathed in 5 ml K-HS (37 ℃) in both sides and gassed with 95%
162 O_2 and 5% CO_2. The transepithelial potential difference for each preparation was
163 measured with the Ussing chamber system (Physiologic Instruments, San Diego, CA,
164 USA; VCC MC6).

165 ***In vivo* permeability measurement**

166 The assay was slightly modified from the previously described methods (Moussaoui
167 *et al.*, 2016). Briefly, the rats were given an oral gavage of fluorescein isothiocyanate
168 (FITC)-dextran (4.4kD) at a final dose of 600 mg/kg in PBS at 9:00 am. After 4 h, a
169 blood sample was taken from each rat by cardiac puncture. The blood was centrifuged
170 at 4 ℃, 3000 ×g for 20 min, and the plasma was taken for the analysis of the
171 FITC-dextran concentration. The plasma was diluted at 1:2 with PBS, and the
172 fluorescence intensity of the diluted plasma was then measured by using a
173 fluoro-spectro photometer (Hitachi Ltd, Tokyo, Japan) with an excitation wavelength
174 of 480 nm and an emission wavelength of 520 nm. The plasma FITC-dextran
175 concentrations were calculated from standard curves generated by serial dilution of
176 FITC-dextran in control plasma.

177 **Statistical analysis**

178 The results are given as arithmetic means ± SEM; "*n*" refers to the number of rats or
179 the number of pairs. Statistical analyses included the Student's paired or unpaired

t-test. Statistics and graphs were generated by using GraphPad Prism, version 5.0 (GraphPad Software, San Diego, Calif., USA). "*p*" values less than 0.05 were assumed to denote a significant difference.

RESULTS

Characterization of the bilateral 6-OHDA lesions

Tyrosine hydroxylase (TH), which is the rate-limiting enzyme of DA synthesis, acts as the most important dopaminergic marker. The results indicated that the TH-immunoreactive dopaminergic neurons in SN and the fibres in the striatum were significantly reduced in 6-OHDA rats (Fig. 1A). Compared to the control rats, the DA contents in the SN and striatum were markably decreased from 63.7 ±6.9 ng/g to 40.4 ±4.9 ng/g (n=8, p<0.05) and from 2270.0 ±181.2 ng/g to 1344.0 ±95.9 ng/g (n=8, p<0.001), respectively (Fig. 1B).

At the fifth week after 6-OHDA treatment, each rat was transferred into an individual metabolic cage to its monitor body mass and daily consumption for one week. No difference in body mass (399.7 ±5.6 g vs. 389.7 ±6.2 g), food (30.2 ±1.3 g vs. 29.1 ±2.9 g) and water consumption (27.4 ±1.2 g vs. 26.6 ±1.3 g) were observed between the control and the 6-OHDA rats (n=12, p>0.05). However, the rotarod test results showed decreased treadmill occupancy times in 6-OHDA rats (n=12, p<0.001) (Fig. 1C), which suggests that the lesion of dopaminergic neurons in the SN caused motor coordination and balance function disorder. As shown in Fig. 1D, the food residue was detected at the bottom of the metabolic cage and collected throughout a 24 h period for measurement. The increased daily food debris of 6-OHDA rats, from

0.3±0.1 ng/g to 3.1±0.2 ng/g (n=12, p<0.001), suggested the emergence of rigidity in the PD model (n=10, p<0.001) (Fig. 1E).

Gastrointestinal motility dysfunction of the 6-OHDA rats

An *in vivo* digital X-ray imaging system was used to evaluate gastric emptying and intestinal transit time. Following a 20 h fast, 3 mL of a barium sulphate suspension was administered to each rat at room temperature. Images were recorded every 45 min after barium meal ingestion. The results indicated that the gastric areas of 6-OHDA rats were significantly larger than those of the control group, but gastric emptying and intestinal barium meal transit were apparently slower in all images throughout the experiment (Fig. 2A). After barium meal intragastric administration for 3 h, the gastric areas were obviously increased from 0.69±0.06 in the control group to 0.96±0.06 in the 6-OHDA group (n=7, p<0.01); however, compared to the control stomach content emptying of 74.11 ±4.37%, only 37.30 ±2.90% of the stomach contents were emptied in the 6-OHDA rats (n=7, p<0.001) (Fig. 2B). Intestinal transit is most often measured as the total intestinal transit time along the entire alimentary tract and is mainly a function of propulsion in the small and large intestine. To confirm the intestinal transit time, the time of the first stool including barium meal was recorded. The 6-OHDA rats produced a longer transit time after barium meal intragastric administration from 339±14 min to 483±26 min (n=8, p<0.001) (Fig. 2C). Furthermore, the number of daily faecal pellets and the faecal content, including solid matter (n=15, p<0.001) and moisture (n=15, p<0.05), were significantly decreased in 6-OHDA rats (Fig.2 D-F), which indicated the impairment of GI motility.

Gastrointestinal barrier dysfunction of the 6-OHDA rats

The FITC-dextran concentration was determined from analysis of the standard curve of dextran-FITC using a 96-well microplate fluorescence reader. Compared with the control rats, the 6-OHDA rats showed increased FITC-dextran permeability from 0.16±0.02 μg/ml to 0.23±0.02 μg/ml by *in vivo* measurement (n=9, p<0.01) (Fig. 3A), whereas intestinal transepithelial resistance (TER) decreased from 46.36±2.82 Ω/cm^2 to 35.38±3.52 Ω/cm^2 (n=12, p<0.05) in the duodenal preparations and from 95.19±4.88 Ω/cm^2 to 62.60±4.89 Ω/cm^2 (n=12, p<0.05) in the colonic preparations (Fig. 3B), which suggested that the intestinal mucosal barrier was impaired. A link between alterations in inflammatory factors and GI dysfunction, especially intestinal permeability, has been reported (Netusha *et al*., 2008). Therefore, inflammatory factors in the plasma were detected. The results showed that the pro-inflammatory factors IL-1β (n=7, p<0.05) and IL-8 (n=7, p<0.01) were significantly increased, and the anti-inflammatory factor IL-10 (n=7, p<0.01) was decreased in 6-OHDA rats.

DISCUSSION

Although the 6-OHDA rats employed in the present study did not mimic all clinical and pathological symptoms of PD patients, the data in the present study provided evidence that this animal model is a useful tool to investigate the mechanisms of Parkinsonian GI disorders. GI dysfunction, together with sleep dysfunction, dysosmia, and other dysautonomia are included in the non-motor symptoms (NMS) of PD, which are key components of PD and present from the ‘pre-motor’ phase to the final palliative stage (Zis *et al*., 2015). Therefore, using the bilateral 6-OHDA model to

study the mechanisms involved in the progression of GI dysfunction in PD would benefit the quality of life in PD patients.

The main neuropathological features of PD are the loss of dopaminergic neurons in the SN and their projections into the caudate nucleus (Zheng *et al.*, 2011). The results from immunohistochemistry and UPLC-MS/MS showed significant depression of the TH-immunoreactive signalling and DA content of the SNs and striata of the 6-OHDA rats. It has been reported that a direct or indirect connection may exist between the SN-striatum and the DMV (Zheng *et al.*, 2011), and DA modulates the neurons in the DMV, which may contribute the impaired gastric motility (Anselmi *et al*., 2017). Furthermore, activating D_1R or D_2R in the DMV can hyperpolarize or depolarize the membrane potential of DMV neurons innervating the GI tract, especially those in the stomach (Zheng *et al*., 2007).

GI dysfunction is often considered an essential PD symptom that dominates the clinical outlook for some patients and is mostly represented by gastroparesis, constipation and peptic ulcers (Jiang P *et al*., 2018; Fornai *et al.* 2016). It has been found that 6-OHDA rats had enhanced expression of dopaminergic markers, which suggests a significant increase of DA content in the guts of 6-OHDA rats (Tian *et al.* 2008). However, increased DA and reduced acetylcholine content in the gastric muscularis externa lead to impaired gastric motility in 6-OHDA rats (Zheng *et al.* 2014). Zhang *et al.* also reported that high DA levels and upregulated D_1 receptors in smooth muscle resulted in an enhanced inhibitory effect on colonic contraction in the cold-restraint stress condition (Zhang *et al.* 2012). The radiological findings regarding

delayed intestinal transit have been previously observed in 6-OHDA unilateral administration rats, which is also due to an impairment of acetylcholine release from colonic myenteric neurons (Fornai *et al.* 2016). In the present study, we applied an *in vivo* digital X-ray imaging system to confirm impaired gastric emptying and intestinal transit time in 6-OHDA rats. The alterations of the enteric neurotransmitters (DA, acetylcholine, nitric oxide, and vasoactive intestinal peptide) involved in the regulation of intestinal motility in the 6-OHDA model suggest that central dopaminergic neurodegeneration is associated with remodelling of enteric neurotransmission (Pellegrini *et al.*, 2016). In contrast, the intraperitoneal MPTP-lesioned mice showed a loss of dopaminergic neurons both in the SN and in the gastric wall (Tian *et al.* 2008; Natale *et al.* 2010), while the MPTP-based animal models showed no significant changes in gastric emptying or intestinal transit time (Anderson *et al.* 2007).

Mucosal barrier damage with high permeability and bowel inflammation plays an important role in peptic ulcer formation (Feng *et al.* 2017). Intestinal permeability can be assessed *in vivo* by determining the permeability of FITC-dextran with a defined molecular size in the blood plasma. TER is another common physiological index used to evaluate the mucosal barrier. Our study showed that 6-OHDA rats had increased FITC-dextran permeability and decreased TER in the gut, thus indicating attenuated mucosal integrity (Monica *et al.* 2015). Moreover, the GI barrier can provide an immune sentinel function by secreting various cytokines in the bacterial stimulation, including the IL-1 family (e.g., IL-1β, IL-18, and IL-33), IL-6, IL-8, and some

anti-inflammatory cytokines (e.g., IL-10 and IL-25). Studies have shown that IL-1β participates in the inflammatory responses by augmenting the infiltration of neutrophils via the activation T cells and innate lymphoid cells (Sun *et al.* 2017). Our results showed that 6-OHDA rats displayed a chronic intestinal disorder, which was caused by an exaggerated immune response with increased pro-inflammatory factors IL-1β and IL-8 and decreased anti-inflammatory factor IL-10. Interestingly, similar results have also been reported by Pellegrini et al. in that there were increased levels of MDA, TNF, and IL-1β in colonic walls isolated from 6-OHDA rats, which suggests the presence of gut inflammation and oxidative stress in the colonic wall (Pellegrini *et al.*, 2016).

In conclusion, our findings suggest that 6-OHDA rats would be an available PD model to investigate the mechanisms involved in the progression of GI non-motor dysfunctions and improve the quality of life of PD patients though drug treatment and more effective assistance.

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FIGURES WITH CAPTIONS

Fig. 1. Characterization of bilateral 6-OHDA lesions.

(A&B) The TH immunoreactivity and DA content in the SNs and striata of control and 6-OHDA rats. (C) The rotarod test results of control and 6-OHDA rats. (D) The original recording showing daily food debris of control and 6-OHDA rats. (E) A summary of the daily food debris of the control and 6-OHDA rats. Values are the means ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$

Fig. 2. Gastrointestinal motility dysfunction of the 6-OHDA rats.

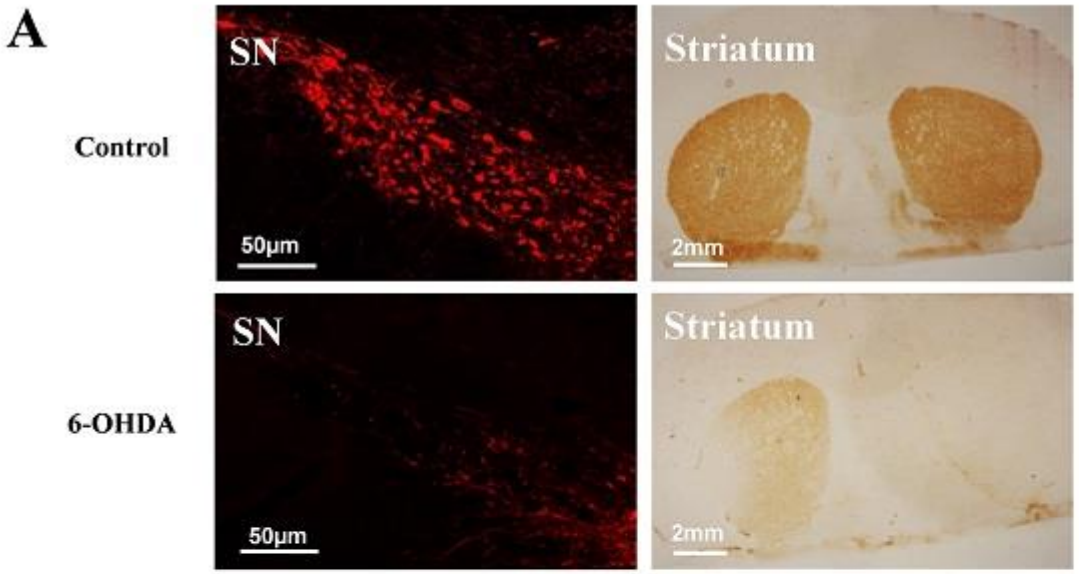
(A) The original images of gastric emptying every 45 min after barium meal ingestion in control and 6-OHDA rats. (B) The gastric area and emptying of the barium meal

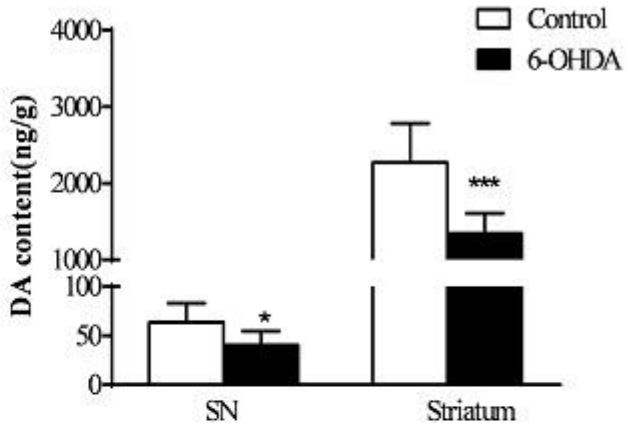
after 3 h in control and 6-OHDA rats. (C) The total intestinal transit time in control and 6-OHDA rats. (D-F) The number of stools and solid matter and moisture content of faeces in control and 6-OHDA rats. Values are means ± S.E.M. $^{*}p$ <0.05, $^{**}p$ <0.01, and $^{***}p$ <0.001.

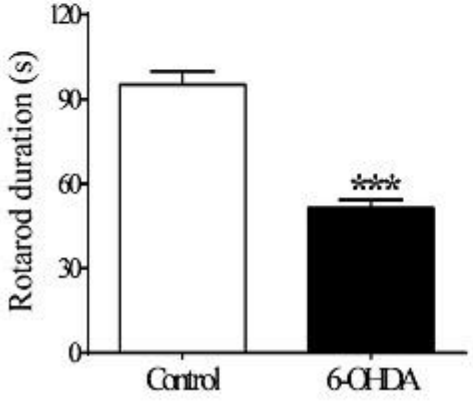
Fig. 3. Gastrointestinal barrier dysfunction of the 6-OHDA rats.

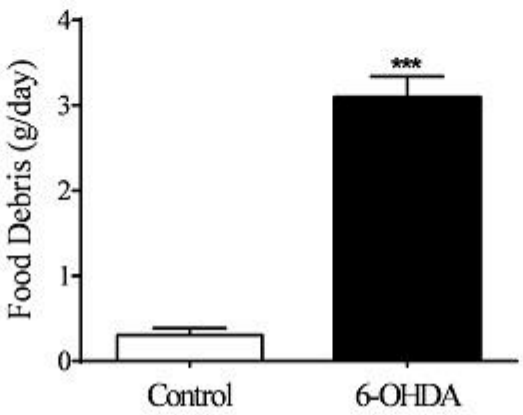
(A) The FITC-dextran concentration in the plasma of control and 6-OHDA rats. (B) The TERs of duodenal and colonic preparations in the control and 6-OHDA rats. (C) The inflammatory factors in the plasma of control and 6-OHDA rats. Values are means ± S.E.M. $^{*}p$ <0.05, $^{**}p$ <0.01, and $^{***}p$ <0.001.

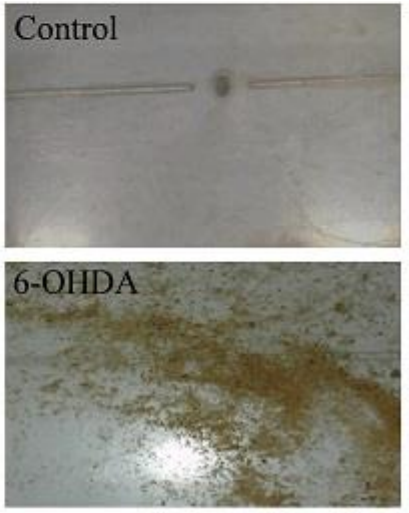
Figures. 1


A
Control
6-OHDA
SN
Striatum
50μm
2mm
SN
Striatum
50μm
2mm

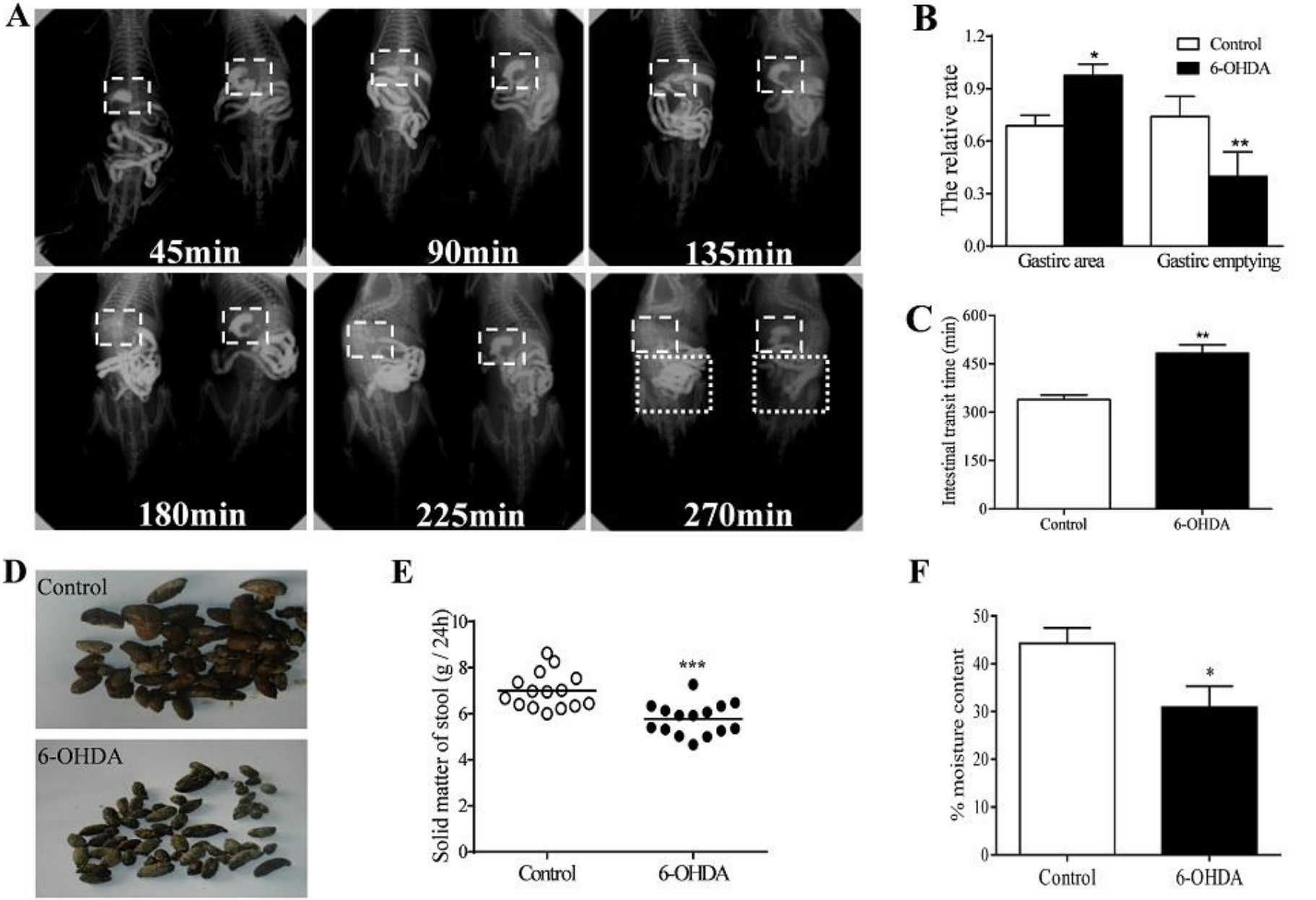

B
DA content(ng/g)
4000
3000
2000
1000
100
50
0
Control
6-OHDA
*
***
SN
Striatum


C
Rotarod duration (s)
120
90
60
30
0
***
Control
6-OHDA

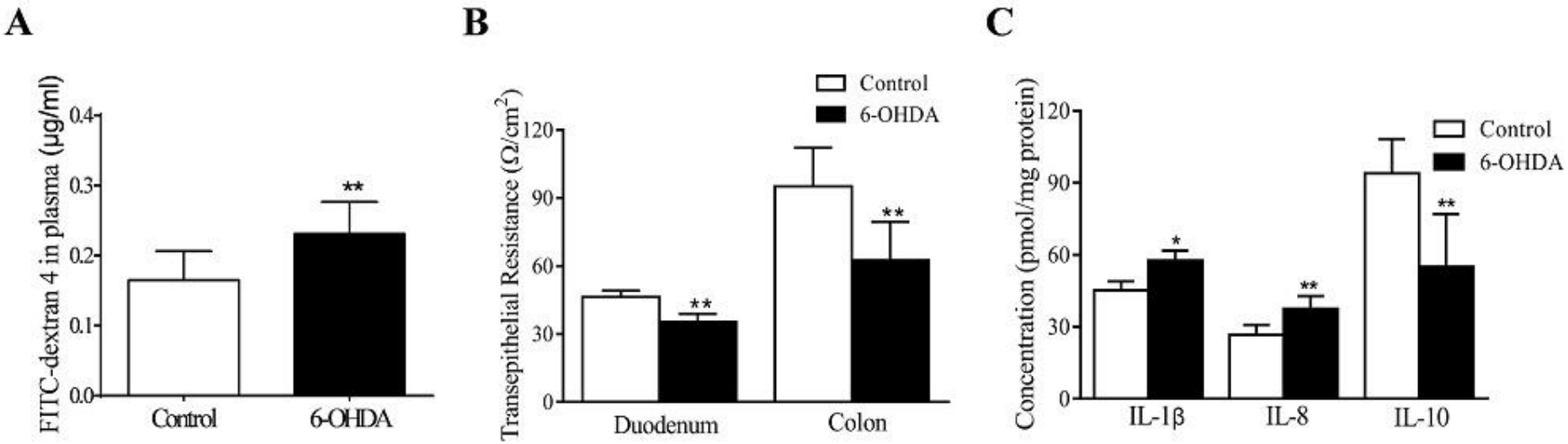

D
Food Debris (g/day)
4
3
2
1
0
***
Control
6-OHDA


E
Control
6-OHDA

Figures. 2


A
45min
90min
135min
180min
225min
270min
B
The relative rate
Control
6-OHDA
Gastirc area
Gastirc emptying
C
Intestinal transit time (min)
Control
6-OHDA
D
Control
6-OHDA
E
Solid matter of stool (g / 24h)
Control
6-OHDA
F
% moisture content
Control
6-OHDA

Figures. 3


A
FITC-dextran 4 in plasma (μg/ml)
0.4
0.3
0.2
0.1
0.0
**
Control
6-OHDA
B
Transepithelial Resistance (Ω/cm²)
120
90
60
30
0
Control
6-OHDA
**
**
Duodenum
Colon
C
Concentration (pmol/mg protein)
120
90
60
30
0
Control
6-OHDA
*
**
**
IL-1β
IL-8
IL-10