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Colonic electrical stimulation improves colonic transit in rotenone-induced Parkinson's disease model through affecting enteric neurons

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ABSTRACT

Aims: The aims of this study were to investigate the effect of colonic electrical stimulation (CES) on delayed colonic transit in Parkinson's disease (PD) model induced by rotenone and its possible mechanisms.
Main methods: Sprague-Dawley male rats were implanted with a pair of electrodes on the serosa at the proximal colon and rotenone was subcutaneously injected for 6 weeks to induce the PD model. Behavior activity, stool volume and open-field test were recorded during the injection. Colonic propulsion rate was measured 6 weeks after rotenone injection. Colon samples of all rats were collected for the measurement of phosphorylated alpha-synuclein, choline acetyltransferase (CHAT), neuronal nitric oxide synthase (nNOS), and tyrosine hydroxylase (TH). The protocols of control rats were the same as the PD rats except that no electrodes were implanted and no rotenone was injected.
Key findings: (1) Rotenone-induced PD rats demonstrated weight loss, significant decrease of the dopaminergic neurons in substantia nigra, and impairment of colon movement. (2) CES significantly accelerated the delayed colonic transmit (91.67 ± 5.58% vs 51.33 ± 4.18%), superior to Macrogol-4000. (3) CES significantly upregulated the expression of CHAT, nNOS and TH protein in colon of PD rats. (4) In colon of PD rats, the phosphorylated alpha-synuclein was significantly upregulated, but CES had no significant effect on phosphorylated alpha-synuclein.
Significance: Our data show that CES can normalize the delayed colonic transit and this normalization may attribute to affecting enteric excitatory and inhibitory neurons.

1. Introduction

Gastrointestinal (GI) dysfunction is a prominent non-motor feature of Parkinson's disease (PD) [1,2]. Of all GI symptoms, constipation is one of the most prominent lower GI dysfunction and pre-motor GI manifestation of PD [3]. According to epidemiology, the prevalence of constipation in PD patients ranges from 24.6% to 63% [4,5]. In addition, people with constipation are at a higher risk of developing PD [6–8], and severe constipation during the course of PD affects absorption of antiparkinsonian drugs which causes aggravation of motor symptoms [9,10]. The pathophysiological findings of the GI motility disorder in PD include prolonged stool transit time, lower amplitude of colonic contraction and dysregulated smooth muscle motor activity [11,12]. The mechanisms of these abnormalities are still not well investigated. Generally, the neuropathological alterations within the enteric nervous system (ENS) could be involved in the GI dysfunction frequently encountered in PD patients [13].

Although GI motility disorders in PD severely reduce the quality of life and aggravate development of the disease, the treatment option is still very poor. For example, *Levodopa* (L-DOPA) treatment is showed to lessen post-defecation residuals, but not improve mean colon transit time (CTT) and bowel movement frequency in patients with PD [14]. Macrogol is recognized to be possibly useful in improving constipation, but recommendations are only level C for patients with PD [15]. Further, Pedrosa Carrasco AJ et al. has ever identified 18 publications involving 15 different interventions for treatment of PD patients with constipation, of which none could be attributed sufficient evidence to derive strong recommendation [16].

Electrical stimulation is a potential effective treatment for refractory GI motility disorders [17]. Previous studies have demonstrated electrical stimulation could improve gastric emptying and normalize intestinal dysrhythmias [18] in models of gastroparesis or intestinal

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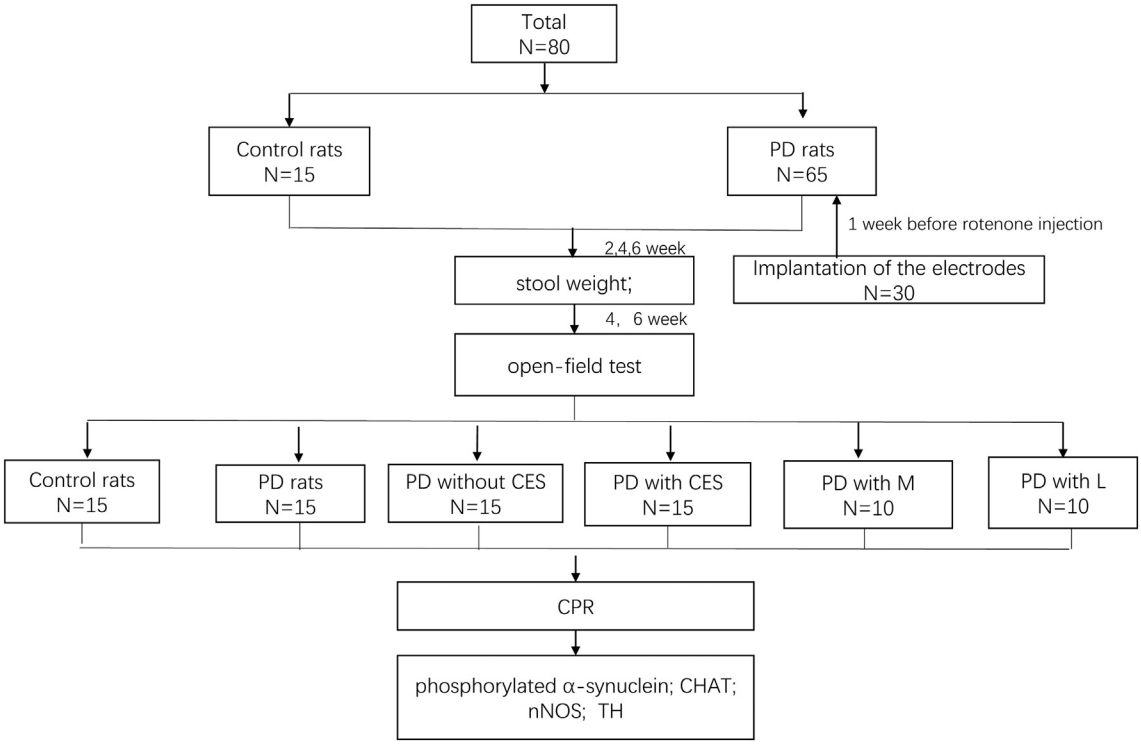

Fig. 1. Study design. Schematic representation of study design. Stool volume and open-field test were performed during the rotenone injection. At 6 weeks, all the rats experienced measurement of colonic propulsion rate. Then, the colon of control, PD, PD without CES, and PD with CES rats was collected for the measurement of phosphorylated alpha-synuclein(phosophorylated α-synuclein), choline acetyltransferase (CHAT), neuronal nitric oxide synthase (nNOS), and tyrosine hydroxylase (TH). PD with M: PD with Macrogol-4000; PD with L: PD with L-DOPA.

dysrhythmia [19,20]. Colonic electrical stimulation (CES) has been shown to enhance colonic transit in normal rats and generate contractions pulse in dog model with delayed colonic transit [21,22]. Although different stimulation parameters and sites have shown effect on GI motility disorders, the effect of CES with pulse trains on the constipation in PD is still unknown.

Therefore, we utilized the rotenone animal model to mimic the constipation and ENS neuropathy observed in PD patients. Then we observed whether CES could improve the constipation and its efficacy. Finally, this study investigated the effect of CES on enteric neuron pathway.

2. Materials and methods

2.1. Animal housing

A total of eighty adult Sprague-Dawley male rats (body weight 300–400 g) were housed at room temperature under a light/dark (12/12 h) cycle. Food and water were provided freely. They were given 1 week for the acclimation upon arrival at the facility. The surgical and experimental procedures were approved by Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology.

2.2. Implantation of the electrodes in the rats

After an overnight fasting, a total of 30 rats were anesthetized by 10% chloral hydrate (0.3 ml/100 g) via intraperitoneal (i.p.) injection. An abdominal midline laparotomy of the rats was performed and a pair of electrodes (COVIDIEN, D7A1042X) was implanted on proximal colon (1 cm distal to the blind colon junction). The electrodes were penetrated the serosa and affixed by unabsorbable sutures. The connecting wires of electrodes were tunneled through the anterior abdominal wall subcutaneously along the right side of the trunk and placed outside the skin back of neck for the attachment to the stimulator [21].

2.3. Treatment

The rats were randomly assigned to a control group and a rotenone-treated PD group. In the control group, the rats were injected only sunflower oil at the dose of 1 ml/kg. In the PD group, the rats were subcutaneously injected by rotenone (2.5 mg/kg/day, sigma, R8875) dissolved in sunflower oil for 6 weeks [23]. Subsequently, the rats in the PD group were randomly assigned to one of the following treatments: (1) PD group; (2) PD group without CES; (3) PD group with CES; (4) PD group with Macrogol 4000; (5) PD group with L-DOPA. The PD group without CES experienced implantation of the electrodes, but without CES. The PD group with CES was composed of trains of short pulses. In PD group with Macrogol 4000, 5 weeks after the rotenone injection, the rats were treated with Macrogol 4000 (10 ml/kg, irrigation) once a day for one week. In L-DOPA-treatment PD group, 4 weeks after rotenone injection, the rats were treated with L-DOPA (25 mg/kg, i.p.) once a day for two weeks [24].

2.4. Demonstration of PD model

General health and gross motor skills were assessed by observing in-cage behavior, such as rigidity (hunched posture and increased tail tone), bradykinesia (slowed movement and/or absence of rearing), and dystonia (clenched paws).

Open-field test was used to evaluate motor coordination of the PD model 4 weeks and 6 weeks after the rotenone injection. The apparatus consisted of a rectangular box (40 × 50 × 63 cm) with a floor divided into 20 (10 × 10 cm) rectangular units. The animals were gently placed in the right corner of the open-field and allowed to freely explore the area for 5 min. Three motor parameters were quantified throughout this

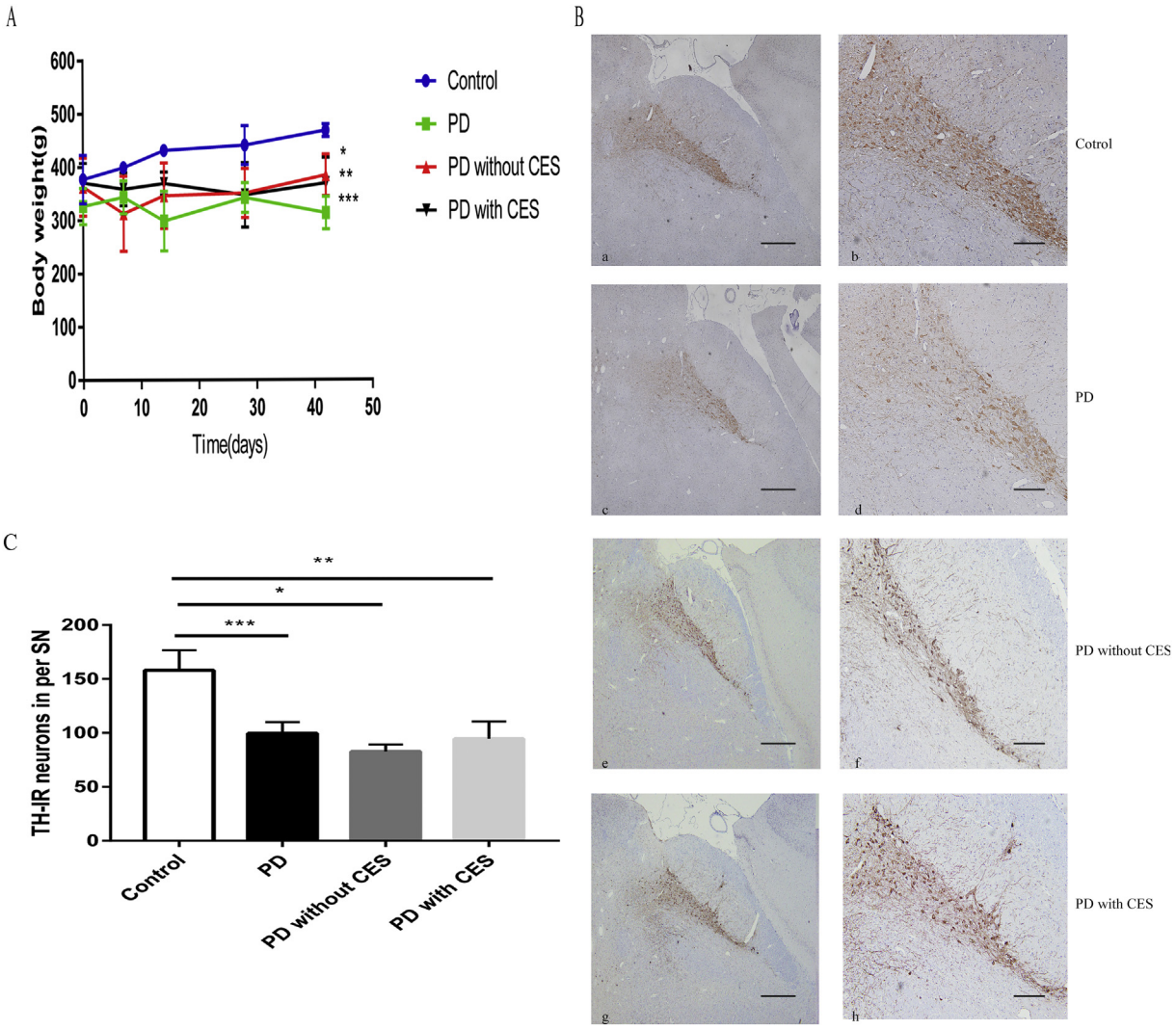

Fig. 2. Development of PD model. (A) The body weight at the indicated time points following rotenone injection (n = 6–9 rats per group). (B) Representative images of Tyrosine hydroxylase (TH) immunoreactive (IR) neurons in the substantia nigra (SN) 6 weeks after rotenone injection (a and b: control group; c and d: PD group; e and f: PD group without CES; g and h: PD group with CES). Scale bar = 500 μm (a, c, e and g) and 200 μm (b, d, f and h). (C) Quantitative analysis of the number of TH-IR neurons in per SN (each group, n = 5–6) 6 weeks after rotenone injection. (A and C) Data are mean ± SEM, by one-way ANOVA with Bonferroni's multiple comparison test. ***P < 0.05 between PD and control rats at 6 weeks; *P < 0.05 between PD without CES and control rats at 6 weeks, **P < 0.05 between PD with CES and control rats at 6 weeks.

Table 1
Open-field test in the control and PD rats.

	Control		PD	
	4 weeks	6 weeks	4 weeks	6 weeks
Locomotion distance (cm)	134.2 ± 14.4	101.1 ± 5.9	123.4 ± 10.3	43.3 ± 11.3**
Rearing frequency	27.8 ± 3.2	20.3 ± 2.3	21.4 ± 1.7*	4.1 ± 2.1**

The locomotion distance and rearing frequency were significantly lower in the PD rats than that in control rats at 6 weeks. Sunflower oil-treated rat (for simplicity, control). Data are mean ± SEM. n = 9 per group.

* P < 0.05 vs. control at 4 weeks.

** P < 0.05 vs. control at 6 weeks.

test: locomotion distance (the number of rats crossing the ground square), rearing frequency (the times of rats standing on their hind paws) and grooming (forelimb neat fur) [25]. The open-field was cleaned with a 75% water-ethanol solution before behavioral testing to eliminate possible bias due to odors left by previous rats.

The dopaminergic neurons in the substantia nigra (SN) were examined by immunohistochemistry. The number of TH-immunoreactive (IR) neurons was counted to evaluate loss of cell bodies in the SN [11]. The rats presenting both motor symptoms and dopaminergic neurodegeneration were considered as successful induction of PD model.

2.5. Evaluation of the severity of constipation and colonic propulsion rate

Fecal samples were collected by metabolic cages at 2, 4 and 6 weeks after the rotenone injection. The colonic propulsion rate (CPR) was measured at the end of modeling (6 weeks after the rotenone injection) to observe and compare colonic transit.

The collection of fecal samples: The rats were transferred into metabolic cages (stool and urine could be separated), in which rats fed and drank freely for normal metabolism. The collection of fecal samples was retained for 12 h. After collection, the changes in fecal form (pellet size) and weight were observed and compared [26].

Propulsive rate evaluation: Following a 12-h fast, every rat was anesthetized by 10% chloral hydrate (i.p.) and the distal ileum was

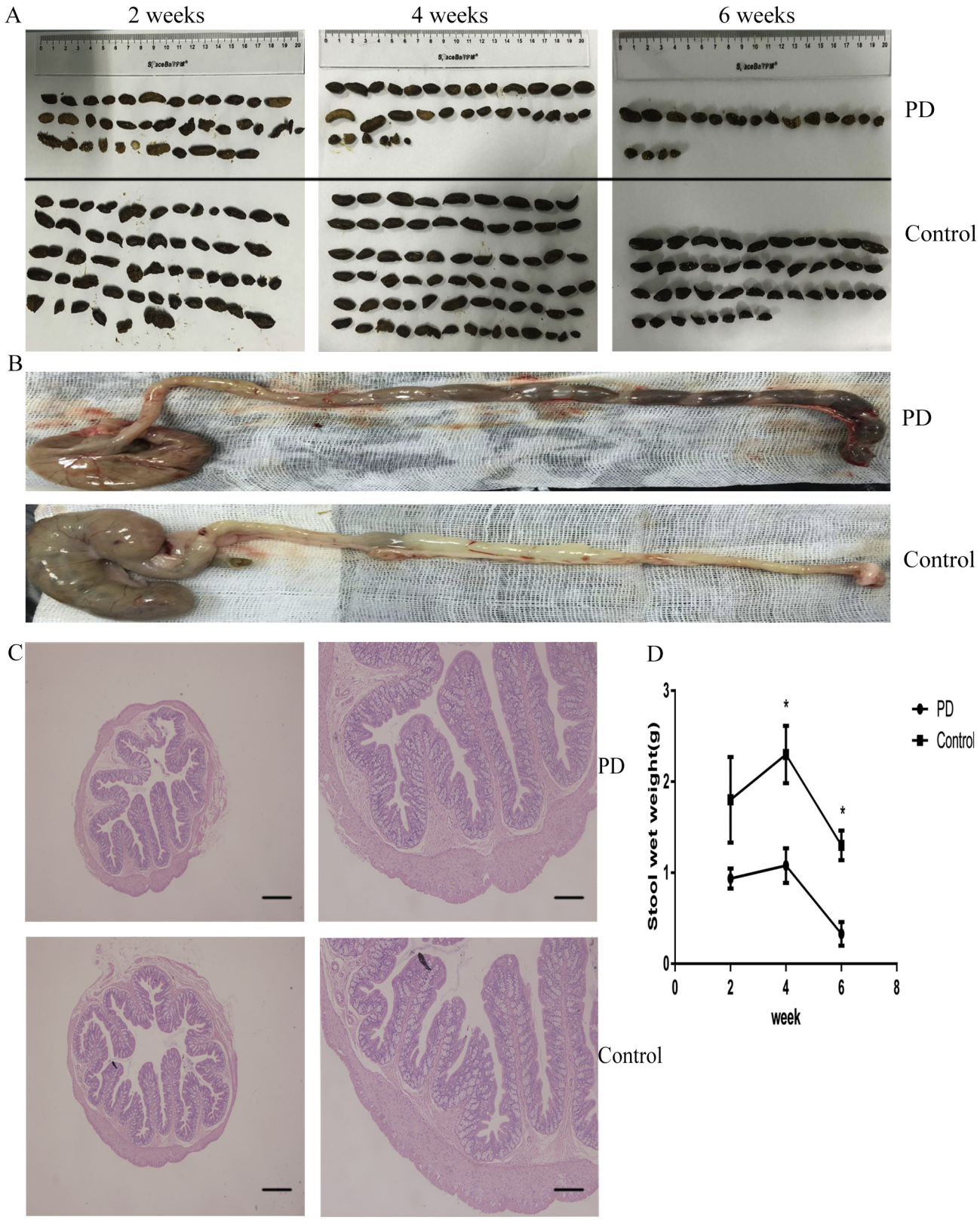

Fig. 3. Constipation in rotenone-induced PD model. (A) The fecal production respectively at 2, 4 and 6 weeks between PD and control rats (n = 9 rats per group). (B) Residual colonic content 12 h after a meal at 6 weeks between PD and control rats. (C) HE staining of colon between PD and control rats at 6 weeks. Scale bar = 500 μm (left) and 200 μm (right). (D) The fecal wet weight at the indicated time points in PD and control rats, *$p < 0.05$ vs control group at 4 weeks and 6 weeks.

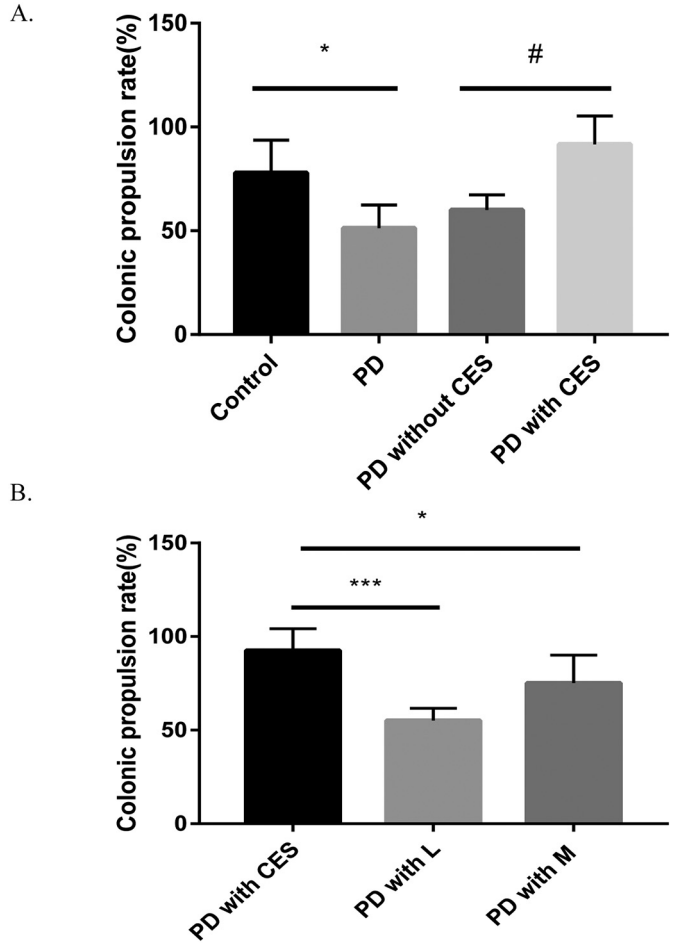

Fig. 4. The effect of CES on colonic transit in rotenone-induced PD model. (A) Colonic propulsion rate of control, PD, PD without CES, and PD with CES rats. *$P < 0.05$ between PD and control rats at 6 weeks, #P < 0.05 between PD with CES and PD without CES rats at 6 weeks. (B) Colonic propulsion rate of PD with CES, PD with L-DOPA, and PD with Macrogol 4000 rats. ***$P < 0.01$ between PD with CES and PD with L-DOPA rats at 6 weeks. *P < 0.05 between PD with CES and PD with Macrogol 4000 rats at 6 weeks. (A and B) Data are mean ± SEM, by one-way ANOVA with Bonferroni's multiple comparison test. PD with M: PD with Macrogol 4000; PD with L: PD with L-DOPA. (n = 4–7 rats per group).

ligated. Suspension (5% charcoal and 10% gum arabic) was infused into blind colon junction. Thirty minutes after the infusion, rats were sacrificed to measure the charcoal moving distance (from the ileocecal valve to the anus) and full-length of the colon [27]. The colonic propulsion rate was calculated by the following formula.

Colonic Propulsion rate

= charcoal moving distance/full − length of the colon

2.6. Electrical stimulation

After 6 weeks of rotenone injection, the 30 rats with implantation of the electrodes were randomly divided into PD group without CES and PD group with CES.

In the PD group without CES, the wire was only connected with the stimulator, but the stimulator was power off. In the PD group with CES, a set of electrical stimulus was composed of trains of short pulses with a train on-time of 2 s and off-time of 3 s, a pulse frequency of 40 Hz, a pulse width of 4 ms, and a pulse amplitude of 10 mA [21]. CES was performed for 40 min before the CPR measurement.

2.7. Tissue preparation for biochemical studies

The tissue preparation was performed exactly after the measure of CPR. Cardiac perfusion was performed by using phosphate-buffered saline (PBS, 0.01 M, pH 7.4) to wash out the blood and then fixed with paraformaldehyde solution (4%). The brains were quickly removed and placed on an ice plate to separate the midbrain for further use. Coronal brain sections were cut at the levels of the SN according to a coronal atlas of the rat brain [28]. Specimens of the colon were excised from rats without perfusion and the contents were washed out with PBS. One part of specimens was kept at −80 °C for use in western blot analysis, and another part was placed in 4% paraformaldehyde for immunostaining.

2.8. Immunostaining

After de-waxing and washing the sections in PBS, antigen retrieval was performed by using boiling citrate buffered saline for 2 min. Sections were cooled to room temperature, and washed with PBS (0.01 M, pH 7.4) and then incubated with blocking reagent (10% normal donkey serum for 1 h). Then the sections were incubated with the primary antibody overnight at 4 °C. For co-immunofluorescence, rabbit primary antibodies against neuronal nitric oxide synthase (nNOS) (1:100, ABclonal), choline acetyltransferase (CHAT) (1:100, ABclonal) and tyrosine hydroxylase (TH) (1:100, GeneTex) were visualized with AlexaFluor488-conjugated goat anti-rabbit secondary antibody (1:200, Invitrogen). Anti-PGP9.5 (1:100, Proteintech) was visualized with AlexaFluor594-conjugated donkey anti-mouse fluorescence secondary antibody. The section was stained by 4′6-diamidino-2-phenylindole for 8 min and coverslipped via antifade mounting medium [29].

For immunohistochemistry staining of TH and Lewy bodies, in brief, sections experienced de-waxing, washing, antigen retrieval, and blocking endogenous peroxidases by incubation with 3% H_2O_2 for 10 min. Then, sections were incubated with phosphorylated at Ser-129 α-synuclein (phosphorylated alpha-synuclein) (1:100, Biolegend) and TH (1:100, GeneTex) antibodies overnight. Finally, sections were incubated with biotin-conjugated goat anti-mouse or anti-rabbit secondary antibody, washed with PBS and exposed to ABC solution for 30 min at room temperature. They were incubated with 3,3′-diaminobenzidine tetrachloride for 2 min, and then washed. Sections were counterstained using hematoxylin, dehydrated and coverslipped.

2.9. Western blot analysis

Full-thickness bowel tissue fragments were homogenized in radioimmunoprecipitation (RIPA) buffer containing 1% protease inhibitor cocktail by tissue homogenizer. The concentration of the supernatant was measured by means of BCA. The concentrations of the protein supernatant aliquots were then equalized based on the results of the Bradford assay by using RIPA buffer. According to the supernatant: protein loading buffer = 4: 1 ratio, the supernatant after centrifugation 120ul and protein loading buffer 30ul denatured at 100 °C and saved at −80 °C. When the target protein were loaded onto an SDS polyacrylamide gel and separated by electrophoresis at 80mv. Proteins were then transferred from the gel to a PVDF membrane at 350 mA. PVDF membrane was blocked with 8% non-fat dry milk or 5% bovine serum albumin (BSA) for 1 h. Subsequently, the membranes were incubated overnight at 4 °C with antibodies, including phosphorylated α-synuclein, TH, choline acetyltransferase (CHAT), and nNos. After washing 3 times in TBST, the membranes were incubated with HRP-linked secondary antibody for 60 min at room temperature. The protein bands were detected using an enhanced chemiluminescence agent (ECL reagents) and analyzed [30].

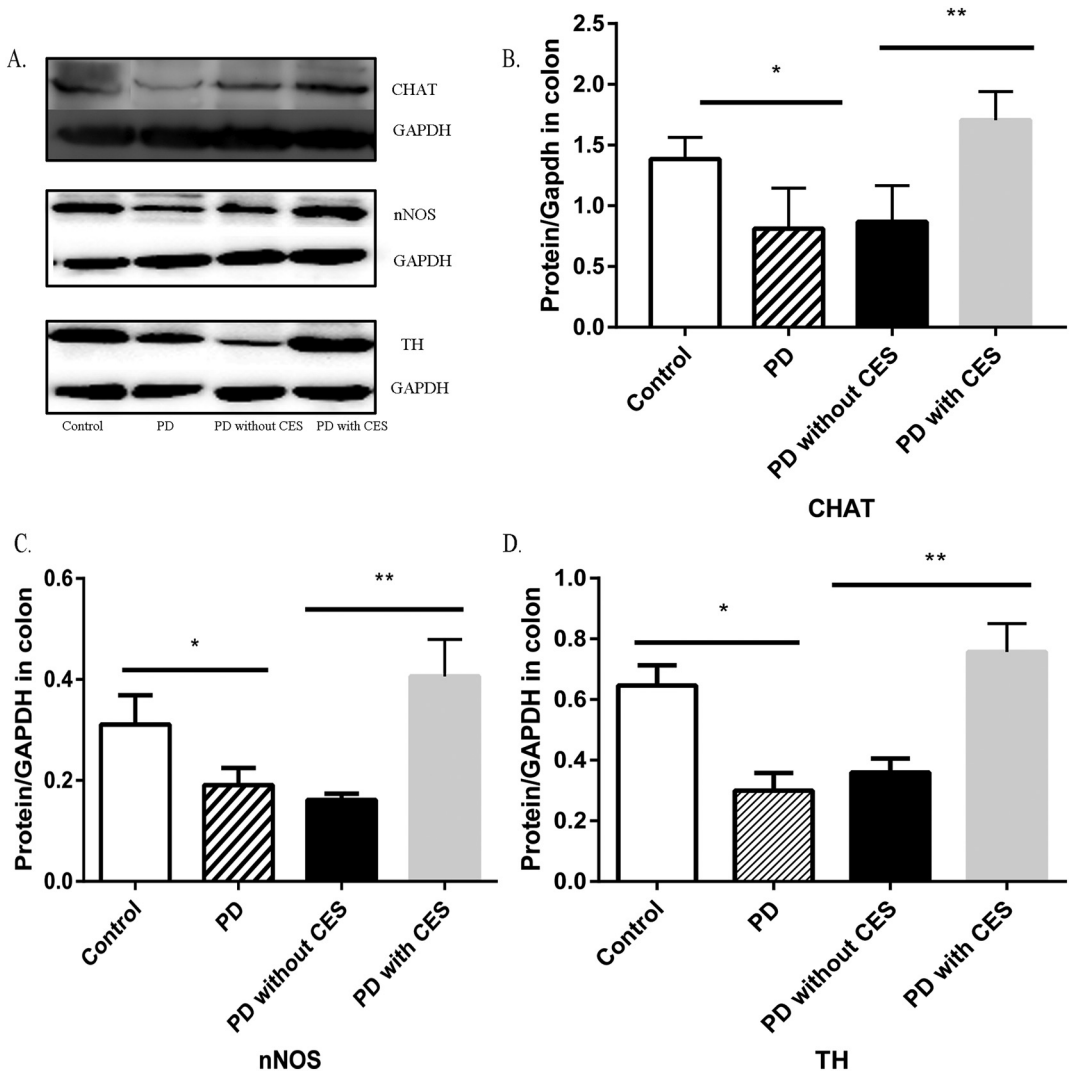

Fig. 5. The effect of CES on ENS in the colon of PD rats. (A) Representative images of western blot analysis of CHAT, TH and nNOS in control, PD, PD without CES, and PD with CES rats at 6 weeks. (B–D) Quantitative analysis of CHAT, TH and nNOS in in control, PD, PD without CES, and PD with CES groups at 6 weeks. (n = 4–6 rats per group). Data are mean ± SEM, by one-way ANOVA with Bonferroni's multiple comparison test. *P < 0.05 between PD and control rats at 6 weeks; **P < 0.05 between PD with CES and PD without CES rats at 6 weeks. (E) Representative micrographs showing immunofluorescent staining of CHAT (green) and a neuronal marker, PGP9.5 (red) in colon at 6 weeks. Nuclei were stained with DAPI (blue). (F) Representative micrographs showing immunofluorescent staining of nNOS (green) and a neuronal marker, PGP9.5 (red) in colon at 6 weeks. Nuclei were stained with DAPI (blue). (G) Representative micrographs showing immunofluorescent staining of TH (green) and a neuronal marker, PGP9.5 (red) in colon at 6 weeks. Nuclei were stained with DAPI (blue). Scale bar = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.10. Study design

The protocol of the study was shown in Fig. 1.

2.11. Statistical analysis

All values were expressed as the mean ± SEM and multiple group comparisons were analyzed by one-way ANOVA analysis of variance, followed by Bonferroni's test. A value of $P < 0.05$ was regarded to be statistically significant. The statistical analysis was performed with SPSS 17.0.

3. Results

3.1. Development of PD model

Rotenone subcutaneous injection successfully induced PD model. During the process of rotenone injection, the mortality rate was 20% at the end of modeling, attributing to paralyzed forelimbs and/or hind limbs, difficulty in walking, and dysphagia. The weight loss was noted in these PD rats, including PD group, PD group without CES, and PD group with CES (Fig. 2A). In the present study, the number of TH-IR neurons in PD rats was significantly lower than that in control group (Control, 158 ± 18.70 vs. PD, 99.50 ± 10.60 vs. PD group without CES, 82.80 ± 6.50 vs. PD with CES, 94.50 ± 16.02) (Fig. 2B, C). Open-field test indicated a decrease in the locomotion distance and the rearing frequency in PD model 6 weeks after rotenone injection (Table 1). The hair color yellowing, bow back, tremor, gait instability and other PD behavioral performance gradually presented 3 weeks after the rotenone injection, which suggested that the autonomous movement of the PD rats decreased, the curiosity weakened, and the ability to explore declined.

3.2. Induction of constipation in rotenone-induced PD model

In rotenone-treated PD rats, constipation was induced during the

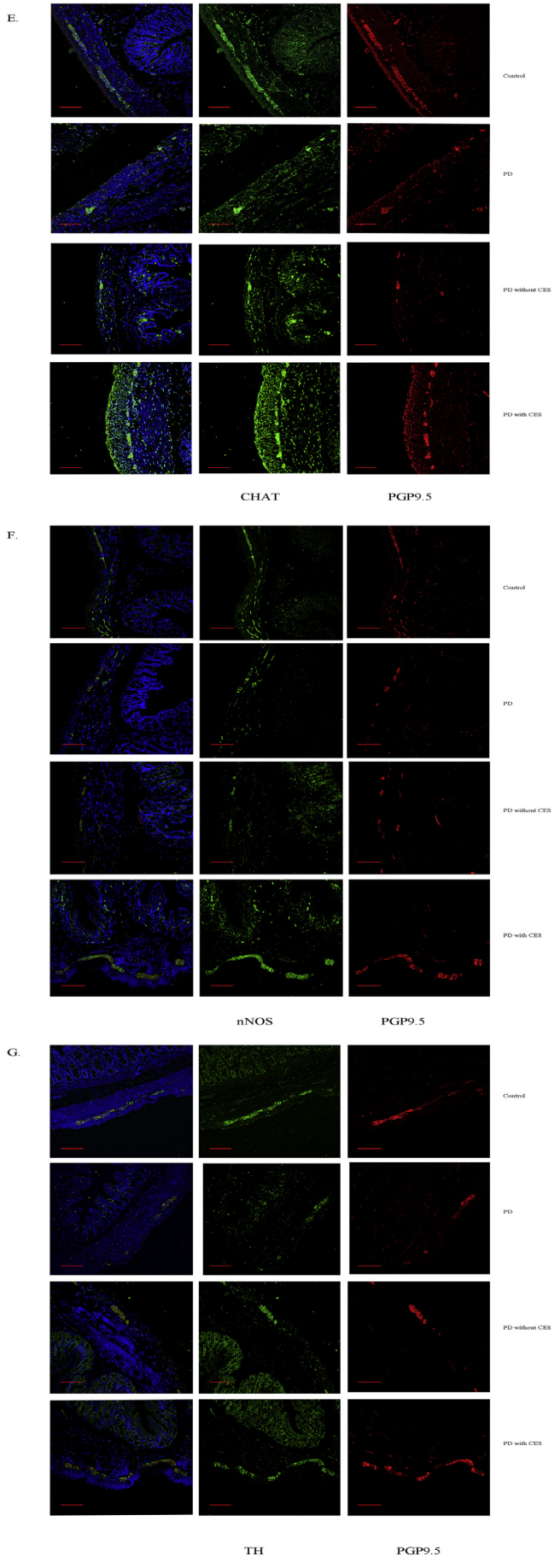

Fig. 5. (*continued*)

rotenone injection. The stool weights were 0.94 ± 0.11 g, 1.08 ± 0.19 g, and 0.33 ± 0.13 g, respectively at 2, 4 and 6 weeks after the rotenone injection. The corresponding stool weights in the control group were 1.80 ± 0.48 g, 2.30 ± 0.32 g, and 1.30 ± 0.16 g, respectively (Fig. 3A,D). The significant difference between the PD and control group was noted at 4 and 6 weeks after the rotenone injection. Furthermore, rotenone-induced PD rats had higher residual colonic content 12 h after a meal and did not appear inflammatory change in colon at 6 weeks, compared with control rats (Fig. 3B,C).

3.3. The effect of CES on colonic transit in rotenone-induced PD model

CES was performed for 40 min. Defecation behavior was presented during the stimulation period and a significantly acceleration of CPR in PD model was noted at 60 min after CES. Within colonic transit test, the percentage was 77.95 ± 6.41% for control group, 51.33 ± 4.18% for PD group, 60 ± 3.63% for PD group without CES, and 91.67 ± 5.58% for PD group with CES (Fig. 4A). Above all, the CPR of PD group with CES was higher than that of PD group with L-DOPA and PD group with Macrogol 4000 (55.17 ± 2.68% for PD group with L-DOPA, and 76 ± 6.38% for PD group with Macrogol 4000) (Fig. 4B).

3.4. The effect of CES on ENS of the colon in rotenone-induced PD rats

CES could accelerate colon transit via activating ENS. The results showed that rotenone exposure reduced the expression of CHAT protein, but treatment with CES markedly enhanced its expression from 0.86 ± 0.14 to 1.70 ± 0.11, compared with PD group without CES (Fig. 5A,B). Immunofluorescence double staining co-localization showed that CHAT was abundantly distributed in myenteric plexus (MP) of control group and PD group with CES (Fig. 5E).

Similarly, rotenone-induced PD rats showed downregulated expression of nNOS protein, compared with control rats. The treatment with CES enhanced the expression of nNOS in colon of PD model from 0.16 ± 0.01 to 0.40 ± 0.07, compared with PD group without CES (Fig. 5A,C). Immunofluorescence double staining co-localization showed that CES significantly upregulated the co-localization for nNOS and the pan-neuronal antibody PGP 9.5 (Fig. 5F).

TH, an enzyme required for dopamine synthesis, decreased in colon of PD model, and CES enhanced its expression from 0.36 ± 0.04 to 0.76 ± 0.09, compared with PD group without CES (Fig. 5A,D). Immunofluorescence double staining co-localization showed that the morphology and density of TH and PGP9.5 for PD group with CES remained near the normal levels or even higher in the colon (Fig. 5G).

3.5. CES has no effect on phosphorylated alpha-synuclein

The protein level of phosphorylated alpha-synuclein in colon of PD model was significantly higher than that in control rats 6 weeks after rotenone injection (Fig. 6A, B). However, there was no significantly difference between PD group without CES and PD group with CES ($P = 0.23$). Phosphorylated alpha-synuclein consisted in cell bodies, which was presented as Lewy bodies (LBs) in myenteric plexus (MP) in PD group, PD group without CES and PD group with CES (Fig. 6C).

4. Discussion

In the present study, we demonstrated that rotenone subcutaneous administration disrupted ENS function and caused delayed colonic transit in rats. The treatment with CES could improve delayed colonic transit, the efficacy of which is superior to Macrogol 4000. CES accelerated colonic transit via upregulating the expression of CHAT, nNOS and TH in colon.

Rotenone, as a lipophilic compound and inhibitor of mitochondrial complex I, could easily cross the blood-brain barrier to reproduce brain pathology of PD [23] and non-motor disorder-GI dysfunction [31].

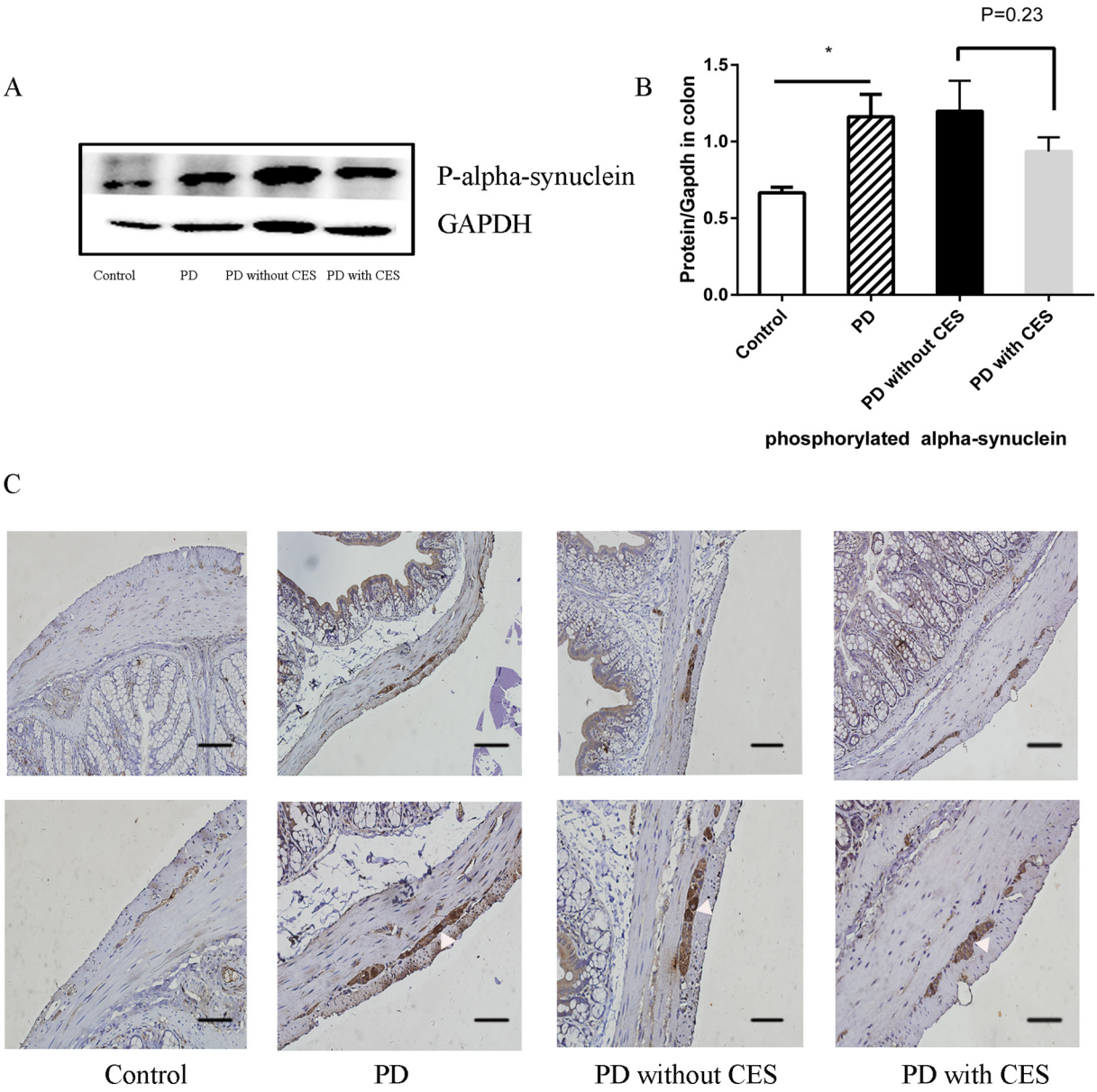

Fig. 6. The effect of CES on phosphorylated alpha-synuclein. (A) Representative images of western blot analysis of phosphorylated alpha-synuclein in control, PD, PD without CES, and PD with CES rats at 6 weeks. (B) Quantitative analysis of phosphorylated alpha-synuclein protein expression (n = 5–7 rats per group). The bars represent the mean ± SEM. *P < 0.05 between PD and control rats at 6 weeks. (C) Representative immunohistochemical images of phosphorylated alpha-synuclein in control, PD, PD without CES, and PD with CES rats at 6 weeks. Triangle represents Lewy body. Scale bar = 100 μm (top) and 50 μm (bottom).

Results of clinical-pathological correlation studies in PD showed that moderate to severe dopaminergic neuronal loss within SN was probably the cause of motor features [32]. In our study, decreased TH-immunostaining neurons at 6 weeks confirmed that rotenone mimicked the brain pathology observed in PD patients. Constipation is a syndrome characterized by colonic and anorectal symptoms. To our knowledge, the effect of rotenone on GI motility focused on the assessment of total GI transit time or gastric emptying [33]. In this study, we focused on the assessment of colonic transit by assessing 12 h stool wet weights and CPR. Our results demonstrated rotenone injection delayed the colonic transit.

Further, we detected the efficacy of CES in accelerating the delayed colonic transmit. L-DOPA has been the mainstay of symptomatic therapy for motor symptoms in PD by maintaining the dopamine concentration at the brain [34]. L-DOPA has been showed to lessen post-defecation residuals in patients [14]. Macrogol 4000, as a non-absorbable osmotic laxative, was reported to relieve the constipation [15]. Therefore, in the present study, L-DOPA and Macrogol 4000 group was set to compare the effect of CES on the constipation in PD. The results showed that the mean CPR remained unaffected by L-DOPA, which suggested that the constipation in PD was more attributed to the peripheral neuropathy. Macrogol 4000 treatment accelerated the colonic transit in the PD rats, but it was not recommended for the longer-term treatment of constipation and lacking of safety concerns for the treatment of constipation in PD clinically [35,36]. Electrical stimulation is an area of great interest and has shown potential effect for treating GI motility disorders. In a long-term clinical investigation, it was reported that lower esophageal sphincter electrical stimulation therapy treated gastroesophageal reflux disease safely and effectively with over a period of 2 years [37]. Similarly, gastric pacing was reported to improve the dyspeptic symptoms in patients with gastroparesis refractory to standard medical therapy. Currently, only one study investigated the effect of colonic pacing on the constipation caused by total colonic inertia, and found that colonic pacing improved rectal evacuation in

66.6% of the patients [38]. In animals' model, CES with trains of short pulses (4 ms) in normal rats and pulse train (6 ms) in normal canine have been demonstrated to accelerate colonic transit [39]. In diphenoxylate/atropine and alosetron-induced dog model of delayed colonic transit, bipolar rectangular pulses, which were generated from four pairs of bipolar electrodes implanted in distal colon, induced colonic contractions pulse [22]. Our data suggested that CES in rotenone-treated PD rats significantly normalized the impaired colonic transit, superior to the Macrogol 4000.

Several studies have demonstrated that lesions in the ENS could be associated with GI dysfunction. It was unclear whether CES accelerated colonic transit via affecting ENS. To explore the effect of CES on ENS, we analyzed the expression of CHAT, nNOS and TH in colon. CHAT, is a rate-limiting enzyme of acetylcholine (ACH) synthesis which is an important stimulating neurotransmitter to contract smooth muscle. Our results showed that in rotenone-induced PD rats, the expression of CHAT was decreased. This result was similar to Fornai's finding which suggested that electrically evoked cholinergic contractions of colonic muscle were decreased and the lowering of colonic transit depended on the impairment of cholinergic enteric neurotransmission in 6-OHDA-induced PD rats [11]. Previous researches also reported that the accelerative effect of CES on colonic transit could be blocked by ACH receptor antagonist [40]. Therefore, our study suggested that delayed colonic transit may be associated with impairment of CHAT in ENS and the CES might accelerate the colonic propulsion via upregulating the expression of the CHAT. Nitric oxide (NO), as an inhibitory neurotransmitter, elicited hyperpolarization and relaxation of GI muscles [41] and was necessary for relaxation of sphincter and muscle during propulsive activity in colon [42]. Previous researches demonstrated the excitatory effect of CES on colon may be mediated via the nitrergic pathway and inhibited by NO blockade. Similarly, the treatment with CES improved CPR via enhancing the expression of nNOS in colon in this study. In addition, Dopamine (DA) is an important monoamine in both brain and gut, inducing inhibitory effects on the circular muscle contractility of mouse distal colon. TH was an enzyme required for DA synthesis, and TH expression was destroyed in brain and gut of PD rats in our result. In line with our results, dopamine neurons in the ENS were destroyed, and colon muscle displayed augmented contraction and impaired relaxation in MPTP-induced PD model [43]. CES could enhance the expression of TH in colon. Given our neuropathological results, we hypothesized neuron dysfunction and death of CHAT, nNOS and TH to be the cause of delayed CRP seen after rotenone injection. CES improved colonic transit via upregulating the expression of CHAT, nNos, and TH.

PD is characterized by DA neuronal degeneration and formation of cytoplasmic LBs or Lewy neurites (LNs) [44]. LBs or LNs, resulting from the quantity abnormality and conformation changing of alpha-synuclein from oligomers, ribbons to fibrils [45], are tightly associated with PD pathogenesis [46]. A recent meta-analysis revealed that the risk of gut alpha-synuclein accumulation in the PD patients was 10.01 fold greater than that in the control patients [47]. LBs or LNs can cause neurotoxicity, and reduce neurotransmitter release and reuptake. A single Ser-129 phosphorylation of alpha-synuclein is the key event responsible for the formation of LBs [48,49]. It's unclear whether CES activates ENS via reducing formation of phosphorylated alpha-synuclein. Our study found that rotenone injection significantly increased protein level of phosphorylated alpha-synuclein in proximal colon, which suggested that rotenone could mimic gut pathology of PD. However, CES didn't reduce the formation of phosphorylated alpha-synuclein.

5. Conclusion

In conclusion, subcutaneous rotenone administration to Sprague-Dawley rat is able to reproduce the clinical symptom of constipation. CES with trains of short pulses accelerates colonic transit and this accelerative effect may be medicated via affecting enteric excitatory and inhibitory neurons. These findings suggest mechanisms involving gastrointestinal motility disorder in PD and a therapeutic potent of CES for PD patients with constipation.

Abbreviations

PD	Parkinson's disease
CES	colonic electrical stimulation
LB	Lewy bodies
LN	Lewy neurites
CPR	colonic propulsion rate
CHAT	choline acetyltransferase
nNOS	neuronal nitric oxide synthase
TH	tyrosine hydroxylase
MP	myenteric plexus
GI	gastrointestinal
P--synuclein	phosphorylated alpha-synuclein
ENS	enteric nervous system
CTT	colon transit time
i.p.	intraperitoneal
SN	substantia nigra
IR	immunoreactive
PBS	phosphate-buffered saline
L-DOPA	*Levodopa*

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Author contributions

JB and JSL conceived the study. JB, and XPH performed the experiments and data analyses. XQ provided intellectual inputs. JB and JSL wrote the manuscript. All authors edited and approved the manuscript.

Declaration of Competing Interest

None.

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