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Original Article

Omega-3 polyunsaturated fatty acids play a protective role in a mouse model of Parkinson's disease by increasing intestinal inducible Treg cells

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Abstract

Parkinson's disease (PD) is defined as a progressive neurodegenerative disease in middle-aged and elderly people. The therapeutic effect of ω-3 PUFAs in several neurodegenerative diseases has been well recognized. Nevertheless, whether nutrition supplementing ω-3 PUFAs exerts a neuroprotective role in PD remains elusive. Bioinformatics revealed 2D chemical structural formula of three components. Mice received indicated treatment with saline, MPTP or ω-3 PUFAs according to grouping. Behavioral function of mice was measured through motor tests such as rearing, akinesia, and rotarod tests. OFT test measured anxiety-like behaviors of mice. Western blotting and TUNEL staining measured dopaminergic fibers and neurons of mice. Western blotting measured inflammation and apoptosis-related protein levels in mouse tissue. FACS measured iTreg cell proportion in colon and brain tissues of mice. ω-3 PUFAs repaired MPTP-stimulated motor function damage in PD mice. ω-3 PUFAs mitigated MPTP-stimulated comorbid anxiety in PD mice. ω-3 PUFAs relieved MPTP-stimulated deficits of dopaminergic fibers and neurons in PD mice. ω-3 PUFAs repressed MPTP-stimulated inflammation and apoptosis pathway activation in PD mice. ω-3 PUFAs repaired MPTP-stimulated immune function damage in PD mice. ω-3 PUFAs exert a protective role in PD mice through alleviating motor function impairment and neuroinflammation by increasing intestinal inducible Treg cells, which may provide a new direction for seeking targeted therapy plans for PD in humans.

Keywords: Microbiota-gut-brain axis, Neuroinflammation, Omega-3 polyunsaturated fatty acids, Parkinson's disease, Treg cells.

1. Introduction

As one of neurodegenerative diseases, Parkinson's disease (PD) is defined as a progressive motor disorder, including motor retardation, rigidity, tremor and severe non-motor disorder [1–3]. PD has a long incubation period, manifested as anosmia, constipation, sleep disorder, etc. [4]. Typical pathological changes include the loss of dopaminergic neurons in the substantia nigra (SN) and striatum (ST) of the brain, and the formation of Lewy bodies in neurons and axons (protein aggregates composed of various proteins such as α-synuclein (α-syn)) [5]. The latest research depicted that α-syn in Lewy bodies expresses in intestinal neurons and endocrine cells, thus some scholars speculated that PD pathology may originate from the intestine [6]. Moreover, when α-syn is injected into the intestine of rodents, it can diffuse into the brain through the vagus nerve, while cutting off the vagus nerve can block its transmission pathway, thereby reducing the risk of PD [7]. These findings link gut microbiota more closely with PD. Similarly, the imbalance of gut microbiota, including qualitative and quantitative changes of gut microbiota, may be closely related to the progression of PD [8].

Due to the complex association of gut microbiota and host, scholars have proposed a new concept: microbiota-gut-brain axis (MGBA), rapidly become a research hotspot in recent years [9, 10]. MGBA refers to a new bidirectional communication pathway between gut microbiota and central nervous system (CNS) [11, 12]. Gut microbiota communicates with the brain through the autonomic nervous system (ANS), vagus nerve, enteric nervous system (ENS), neurotransmitter, immune system, etc. [13]. It has been demonstrated that α-syn aggregates may be transmitted to the brain through MGBA. Moreover, elevation of intestinal permeability has a marked relation to endotoxin, oxidative stress and α-syn aggregates [14]. Additionally, incorrectly folded α-syn can not only activate microglia, but also secrete tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, etc.; it can also facilitate differentiation, cloning and proliferation of primitive T lymphocytes into effector T lymphocytes, and induce microglia to produce neurotoxicity through blood-brain barrier [15, 16]. Thus, gut nutrition support for the brain may be an effective treatment for PD.

Omega-3 polyunsaturated fatty acids (ω-3 PUFAs) are essential fatty acids for the human body, which cannot be synthesized in large quantities in the human body and must be obtained from food [17]. Alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are three vital components of ω-3 PUFAs [18]. The therapeutic effect of ω-3 PUFAs in neurodegenerative

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diseases has been well recognized [19, 20]. Nevertheless, whether nutrition supplementing ω-3 PUFAs exerts a neuroprotective role in PD remains elusive.

The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is usually applied to induce PD characteristics in animal experiments [21]. Thus, we attempted to elucidate role and action mechanism of ω-3 PUFAs in MPTP-stimulated PD mice, which may provide a novel insight for seeking targeted therapy plans for PD.

2. Materials and methods

2.1. Bioinformatics

PubChem website (https://PubChem.ncbi.nlm.nih.gov/) demonstrated 2D chemical structural formula of three components (ALA, EPA and DHA).

2.2. Animals

All male C57BL/6 mice (7 weeks old; weight, 23-25 g; Charles River, MA, USA) were kept housed under a 12/12 h light/dark cycle (24 ± 2°C), free access to food and water, for at least one week before modeling. The Animal Ethics Committee of our hospital granted approval for this research (Approval Number: NB20230321433).

2.3. Modeling

The mice received intraperitoneal injection with saline or MPTP (30 mg/kg, dissolved in saline; Sigma-Aldrich) for 5 consecutive days and were assigned to three groups: Control group (n = 8), MPTP group (n = 8), and MPTP + ω-3 PUFAs group (n = 8). The mice in MPTP + ω-3 PUFAs group received a diet enriched in ω-3 PUFAs (7.75 g/kg) after 5 consecutive days of MPTP injection.

2.4. Behavioral tests

At 12 weeks after MPTP injection, behavioral tests were conducted. Rearing test detected motor function of mice, which counted the number of forelimb wall contacts. The akinesia test detected no body movement in mice. Rotarod test detected motor mediation and balance impairment through a rod instrument (MED Associates, Inc., USA).

2.5. Open field test (OFT)

Anxiety in PD was measured through OFT. Mice were kept housed in a rectangular white acryl box (40 × 40 × 27 cm) individually. Then mouse behaviors (total distance, central zone distance, and numbers of crossing the central zone) received recording for 5 min via a video camera and monitoring through a computerized video-tracking system running the S-MART (Pan Lab Co., Barcelona, Spain).

2.6. Western blotting

Twelve weeks after MPTP injection, the mice received perfusion of 0.2 M phosphate buffer and followed with cold 10% formalin. The mouse brain was harvested and cryo-protected. Total protein was extracted from brain tissue using RIPA buffer containing protease and phosphatase inhibitors. The proteins were separated by SDS-PAGE and then transferred to PVDF membranes. Primary antibodies used in this research include anti-tyrosine hydroxylase (TH), anti-α-syn, anti-glial fibrillary acidic protein (GFAP), anti-Iba-1, anti-TNF-α, anti-IL-1β, anti-Bax, anti-cleaved-caspase-3 and anti-β-actin as well as horseradish peroxidase (HRP)-conjugated secondary antibodies were provided by Abcam. Protein bands got visualization and detection through enhanced chemiluminescence.

2.7. TUNEL staining

The mouse brain tissues embedded in paraffin received incubation with recombinant terminal deoxynucleotidyl transferase (rTdT) solution for 1 h, followed by nuclei staining using DAPI. TUNEL-stained cells were observed through fluorescence microscopy (Olympus).

2.8. Fluorescence-activated cell sorting (FACS)

To measure iTreg ($CD3^+CD4^+CD25^+FoxP3^+$) cell proportion, intracellular FoxP3 staining was conducted with a Mouse Regulatory T Cell Staining Kit (eBioscience) under the manufacturer's guidance. A total of 1×10^6 lymphocytes isolated from colon or brain tissue received surface-staining using anti-CD4-FITC and anti-CD25-APC, followed by fixation and permeabilization using Cytofix / Cytoperm buffer (BD PharMingen, San Diego, CA, USA) and intracellular staining using Phycoerythrin (PE) conjugated anti-Foxp3 or IgG2a. The $CD25^+Foxp3^+$T cells received analysis after gating on $CD4^+$ cells through flow cytometry.

2.9. Statistical analysis

Data were expressed as the mean ± standard deviation of three independent assays. Statistical analysis was conducted using GraphPad Prism 7 software. Comparisons between two groups were assessed with Student's t-test and comparisons among multiple groups were assessed with one-way ANOVA. Each assay was performed for three times. A statistical significance was presented upon $P<0.05$.

3. Results

3.1. ω-3 PUFAs improve motor functions in MPTP-stimulated mouse model of PD

In recent years, ω-3 PUFAs have been revealed to reduce the risk of PD. Thus, we attempted to elucidate the specific role of ω-3 PUFAs underlying PD. Previously, ALA, EPA and DHA have been demonstrated as three vital components of ω-3 PUFAs [18]. Through PubChem, we obtained the same results of components and their 2D chemical structural formula (Figure 1A). To mimic PD characteristics, we established PD mouse models through MPTP stimulation. The mice received intraperitoneal injection with saline or MPTP (30 mg/kg) for 5 consecutive days and were assigned to three groups: Control group (n = 8), MPTP group (n = 8), and MPTP + ω-3 PUFAs group (n = 8); mice in MPTP + ω-3 PUFAs group received a diet enriched in ω-3 PUFAs (7.75 g/kg) after 5 consecutive days of MPTP injection; motor behavioral tests such as rearing, akinesia and rotarod tests were conducted 11 and 12 weeks after injection (Figure 1B). As a result, for the rearing test on forelimb wall contacts, MPTP led to a marked depletion of rear amount and ω-3 PUFAs countervailed this influence (Figure 1C); for akinesia test on no body movement, MPTP caused an evident elevation of latency time and ω-3 PUFAs reversed this trend (Figure 1D); for rotarod test on motor mediation and balance impairment, MPTP resulted in a remarkable downregulation of latency time and ω-3 PUFAs reversed this trend (Figure 1E). Collectively, ω-3 PUFAs repair motor function damage in MPTP-stimulated PD mice.

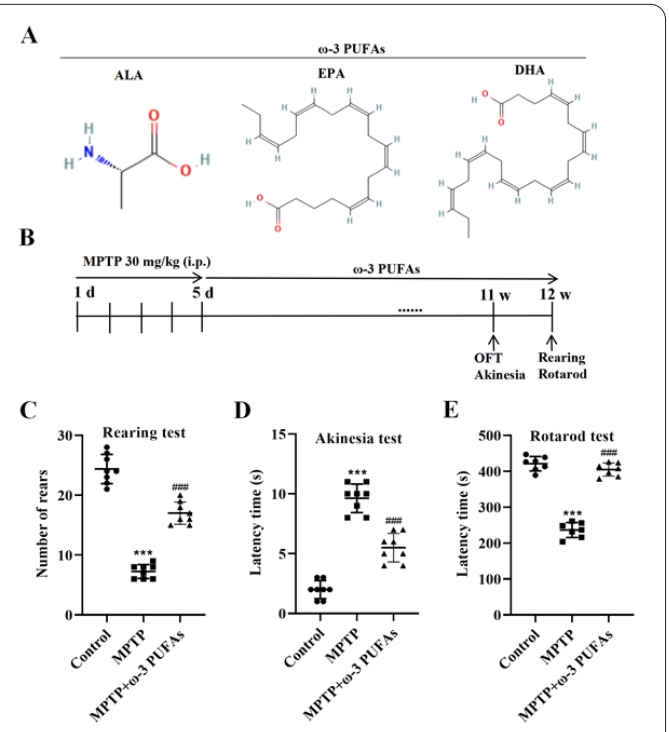

Fig. 1. ω-3 PUFAs improved motor functions in MPTP-stimulated mouse model of PD. A: PubChem website demonstrated 2D chemical structural formula of three components (ALA, EPA and DHA). B: A flow chart of experimental process. C-E: Motor tests such as rearing, akinesia and rotarod tests measured behavioral function of mice in Control (n = 8), MPTP (n = 8) or MPTP + ω-3 PUFAs group (n = 8).

3.2. ω-3 PUFAs ameliorate comorbid anxiety in MPTP-stimulated mouse model of PD

We attempted to clarify ω-3 PUFAs impact on anxiety, one of the non-motor features, underlying PD through OFT. As a result, tracking routes of mice in Control, MPTP or MPTP + ω-3 PUFAs group appeared as shown in Figure 2A. Moreover, total distance presented no difference among the three groups (Figure 2B). MPTP resulted in a marked decline in central distance, central/total distance ratio and amount of crossings in the central zone while all these influences were neutralized by ω-3 PUFAs (Figures 2C-E). Collectively, ω-3 PUFAs mitigate comorbid anxiety in MPTP-stimulated PD mice.

3.3. ω-3 PUFAs upregulate TH expression and reduce α-syn accumulation in ST and SN in MPTP-stimulated mouse model of PD

We attempted to clarify ω-3 PUFAs influence on dopaminergic fibers and neurons. First, we measured TH and α-syn protein abundances in ST of mice through western blotting. As a result, TH protein level presented downregulation whereas α-syn protein level displayed upregulation in ST of mice under MPTP while showing opposite trends under ω-3 PUFAs (Figure 3A). Furthermore, we measured mouse neuronal apoptosis through TUNEL staining. We discovered that TUNEL-positive neuron amount presented upregulation in MPTP mice whereas presented downregulation in MPTP + ω-3 PUFAs mice (Figure 3B). Then, we repeated western blotting and TUNEL staining in SN of mice. The results depicted that TH protein level presented downregulation whereas α-syn protein level displayed upregulation in SN of mice under MPTP while showed opposite trends under ω-3 PUFAs (Figure 3C); TUNEL-positive neuron amount presented upregulation in MPTP mice whereas presented downregulation in MPTP + ω-3 PUFAs mice (Figure 3D). Collectively, ω-3 PUFAs relieve deficits of dopaminergic fibers and neurons in MPTP-stimulated PD mice.

3.4. ω-3 PUFAs suppress inflammation and apoptosis in ST and SN of PD in MPTP-stimulated mouse model of PD

As well known, GFAP, a type III intermediate filament protein, is a vital marker for mature astrocytes [22], while Iba-1 is a sensitive marker for microglia activation [23], and abnormal GFAP and Iba-1 expression occurs in neuroinflammation [22, 23]. Thus, to elucidate and clarify ω-3 PUFAs effect on activation of inflammation and apoptosis pathways, we measured abundances of GFAP, Iba-1, proinflammatory cytokines (TNF-α and IL-1β) and apoptosis-related proteins (Bax and cleaved caspase-3) in ST and SN of mice through western blotting. As a result, MPTP upre-

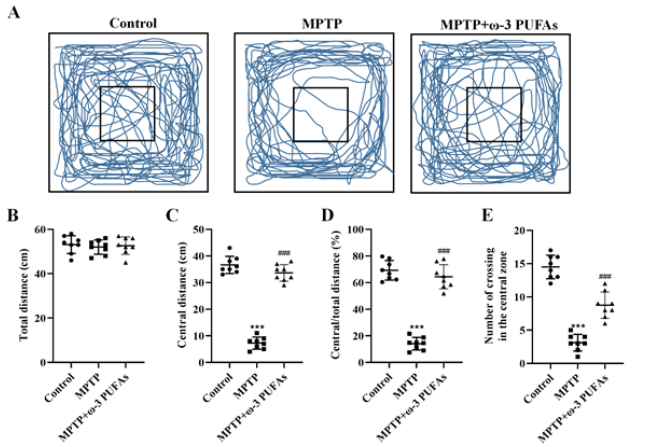

Fig. 2. ω-3 PUFAs ameliorated comorbid anxiety in MPTP-stimulated mouse model of PD. A: OFT test measured anxiety-like behaviors of mice. Tracking routes of mice in Control, MPTP or MPTP + ω-3 PUFAs group. B: Total distance of mice under indicated treatment. C: Central distance of mice under indicated treatment. D: Central/total distance ratio of mice under indicated treatment. E: Number of crossings in the central zone of mice under indicated treatment.

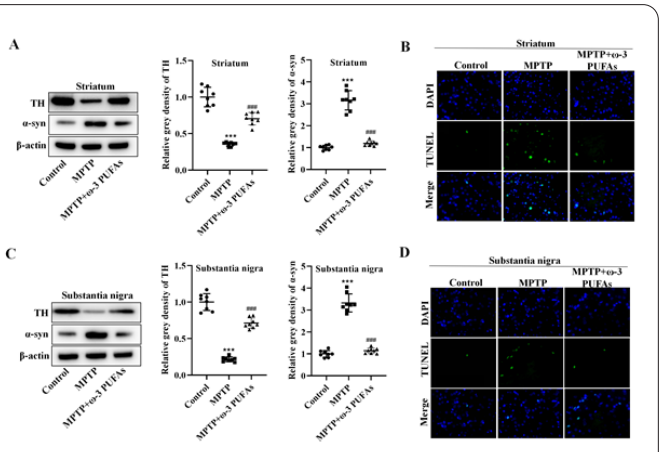

Fig. 3. ω-3 PUFAs upregulated TH expression and reduced α-syn accumulation in ST and SN in MPTP-stimulated mouse model of PD. A: Western blotting measured TH and α-syn levels in ST of mice in Control, MPTP or MPTP + ω-3 PUFAs group. B: TUNEL staining measured apoptosis in ST of mice under indicated treatment. C: Western blotting measured TH and α-syn levels in SN of mice under indicated treatment. D: TUNEL staining measured apoptosis in SN of mice under indicated treatment.

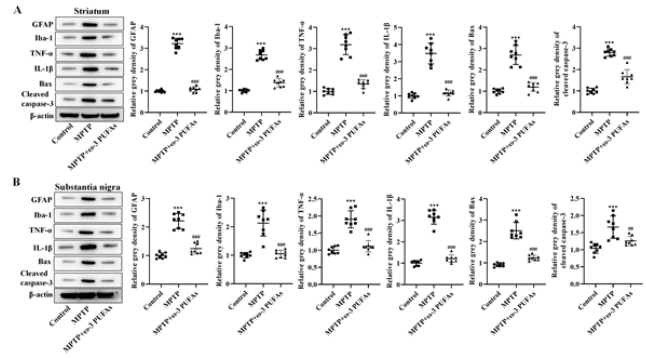

Fig. 4. ω-3 PUFAs suppressed inflammation and apoptosis in ST and SN of PD in MPTP-stimulated mouse model of PD. A: Western blotting measured GFAP, Iba-1, TNF-α, IL-1β, Bax and cleaved caspase-3 protein levels in ST of mice in Control, MPTP or MPTP + ω-3 PUFAs group. B: Western blotting measured GFAP, Iba-1, TNF-α, IL-1β, Bax and cleaved caspase-3 protein levels in SN of mice under indicated treatment.

gulated GFAP, Iba-1, TNF-α, IL-1β, Bax and cleaved caspase-3 protein levels in ST and SN of mice whereas ω-3 PUFAs downregulated levels of these proteins in ST and SN of PD mice (Figures 4A, B). Collectively, ω-3 PUFAs repress inflammation and apoptosis pathway activation in MPTP-stimulated PD mice.

3.5. ω-3 PUFAs increase the percentage of iTreg cells in colon and brain in MPTP-stimulated mouse model of PD

Previously, abnormal immune responses due to Treg cell imbalance had a close relation to PD pathogenesis [24]. Thus, to elucidate and clarify ω-3 PUFAs role in underlying immune function in PD, we measured the proportion of iTreg cells ($CD3^+CD4^+CD25^+FoxP3^+$)/$CD3^+CD4^+$T cells isolated from colon and brain tissues using FACS. As a result, MPTP led to a remarkable proportion decrease of iTreg/$CD3^+CD4^+$T cells in mouse colon and brain tissues and then this change was reversed by ω-3 PUFAs (Figures 5A, B). Collectively, ω-3 PUFAs repair immune function damage in MPTP-stimulated PD mice.

4. Discussion

MGBA is "bidirectional", perfectly integrating the host gut and brain activities, that is, the brain improves and affects gut physiology and composition of symbiotic microbiota through modulating gastrointestinal and immune functions [25]. So far, it has been demonstrated that the rodent brain can regulate visceral sensitivity, gut movement, secretion, permeability, etc.; gut microbiota act on the brain through neuroactive compounds [26]. Microbiota can affect host's neurophysiological changes through producing chemicals that bind to receptors inside and outside the host's intestine. For instance, Bravo et al. have depicted that anxiety-like and depressive behaviors of rats fed with rhamnosus (JB-1) were reduced, and changes in GABA Aα2 mRNA levels in the brain controlling specific behavioral areas were discovered [27]. Short-chain fatty acids (SCFAs) are fermented by gut microbiota with dietary fiber in the large intestine; in animal models, SCFAs can improve the cognitive function of animals with neurodevelopmental and neurodegenerative diseases [28]. Thus, herein, we clarified the effect of intestinal nutrition support on the brain in PD.

MPTP possesses high lipid solubility and is easy to pass through the blood-brain barrier; MPTP is converted into the effective ingredient 1-methyl-4-phenylpyridine (MPP+) under the action of monoamine oxidase in glial cells, selectively destroying the dopaminergic neurons in the SN of mesencephalon, and producing symptoms, progressive processes and pathological changes similar to PD [29, 30], which is an ideal model for replicating human PD characteristics. Herein, we chose MPTP-induced mice as research models. MPTP triggered motor function impairment, enhanced comorbid anxiety and caused deficits of dopaminergic fibers and neurons. Moreover, MPTP triggered neuroinflammation and apoptosis. These findings suggested the successful construction of PD mouse models through MPTP.

DHA-derived molecules through phospholipase on the cell membrane, such as neuroprotectin D1 and resolvin, exert a protective role on nerve cells and elevate anti-apoptotic proteins and anti-inflammation [31, 32]. DHA can improve the function of the blood-cerebrospinal fluid barrier at the cell membrane level, and its mechanism may have a relation to dopamine receptor-mediated neurotransmission [33]. Accumulating evidence has depicted the impact of EPA and terrestrial phospholipids on ameliorating mood disorders; EPA-enriched phospholipids possess higher efficacy than EPA-enriched ethyl ester in mitigating anxiety-like behaviors [34]. Herein, through PubChem website, we discovered that ω-3 PUFAs included ALA, EPA and DHA. Epidemiology has previously depicted that ω-3 PUFAs can reduce the risk of PD; when unsaturated fatty acids in the diet are replaced by saturated fatty acids, risk factors for PD increases; similarly, long-term consumption of fish oil, fruits and vegetables reduces the risk of PD, and long-term consumption of ω-3 PUFAs can also reduce the occurrence of PD complications [35, 36]. Herein, ω-3 PUFAs repaired motor function damage, mitigated comorbid anxiety and relieved deficits of dopaminergic fibers and neurons in MPTP-stimulated PD mice. ω-3 PUFAs also repressed inflammation and apoptosis pathway activation in MPTP-stimulated PD mice. These

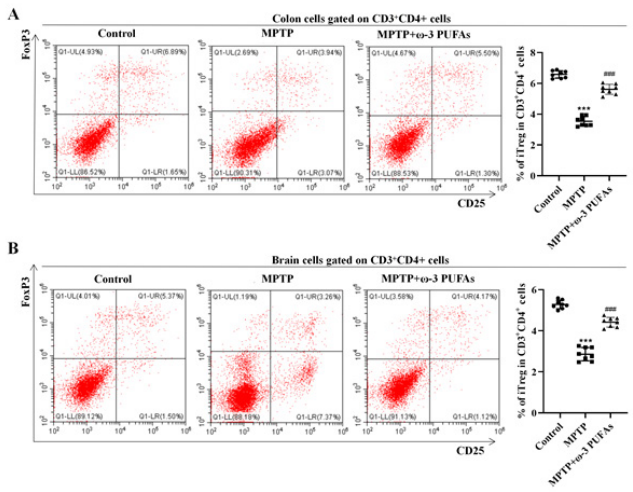

Fig. 4. ω-3 PUFAs increased percentage of iTreg cells in colon and brain in MPTP-stimulated mouse model of PD. A: FACS measured iTreg cell proportion in colon tissue of mice in Control, MPTP or MPTP + ω-3 PUFAs group. B: FACS measured iTreg cell proportion in brain tissue of mice under indicated treatment.

findings suggested that ω-3 PUFAs exert a protective role against PD, which is consistent with previous reports.

$CD4^+$T cells can differentiate into T helper 1 cell (Th1), T helper 1 cell (Th2), Th17 cells and regulatory T (Treg) cells in different cytokine environments; under certain conditions, subsets can be transformed into each other, so that immune effect and immunosuppression of the body can keep a delicate and complex balance [37]. Th17 cells and Treg cells exist simultaneously in multiple tissues, and Th17 cells mainly mediate inflammation and autoimmune diseases, whereas Treg cells exert anti-inflammation and maintain autoimmune tolerance [38]. The transcription factor forkhead box p3 (Foxp3) specifically expresses in Treg cells, which can upregulate cytokines such as TGF-β, IL-10, etc., participating in the inhibitory function of Treg cells, thus exerting an immunoprotective role [39, 40]. Herein, ω-3 PUFAs elevated iTreg/$CD3^+CD4^+$T cell proportion in MPTP-stimulated PD mouse colon tissue and it exerted same effect in MPTP-stimulated PD mouse brain tissue, suggesting ω-3 PUFAs repair immune function damage in MPTP-stimulated PD mice.

In conclusion, ω-3 PUFAs exert a protective role in PD mice through alleviating motor function impairment and neuroinflammation by increasing intestinal inducible Treg cells, providing a new direction for seeking targeted therapy plans for PD in humans.

Informed consent
The authors report no conflict of interest.

Availability of data and material
We declared that we embedded all data in the manuscript.

Authors' contributions
YQ, XY and LY designed the experiments. LX and WY helped with the animal experiments. XY, ZY and LY performed the data analyses. WY and YQ helped perform the analysis with constructive discussions. XY wrote the draft manuscript.YQ, ZY and LY revised the manuscript.

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