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Preoperative gut microbiota of POCD patients induces pre- and postoperative cognitive impairment and systemic inflammation in rats

Xin Wei[1], Fei Xing[1], Yaowei Xu[1], Fan Zhang[1], Dan Cheng[1], Yinhui Zhou[1], Fei Zheng[2] and Wei Zhang[1*]

Abstract

Background Postoperative cognitive dysfunction (POCD) is common following surgery in elderly patients. The role of the preoperative gut microbiota in POCD has attracted increasing attention, but the potential underlying mechanisms remain unclear. This research aimed to investigate the impact of the preoperative gut microbiota on POCD.

Methods Herein, we analyzed the preoperative gut microbiota of POCD patients through a prospective specimen collection and retrospective blinded evaluation study. Then, we transferred the preoperative gut microbiota of POCD patients to antibiotic-treated rats and established POCD model by abdominal surgery to explore the impact of the preoperative gut microbiota on pre- and postoperative cognitive function and systemic inflammation. The gut microbiota was analyzed using 16S rRNA sequencing analysis. The Morris water maze test was performed to evaluate learning and memory abilities. The inflammatory cytokines TNF-α, IL-1β and IL-6 in the serum and hippocampus were measured by ELISA. Microglia were examined by immunofluorescence staining for Iba-1.

Results Based on the decrease in the postoperative MMSE score, 24 patients were identified as having POCD and were matched with 24 control patients. Compared with control patients, POCD patients exhibited higher BMI and lower preoperative MMSE score. The preoperative gut microbiota of POCD patients had lower bacterial richness but a larger distribution, decreased abundance of *Firmicutes* and increased abundance of *Proteobacteria* than did that of control patients. Compared with rats that received preoperative fecal samples of control patients, rats that received preoperative fecal samples of POCD patients presented an increased abundance of *Desulfobacterota*, decreased cognitive function, increased levels of TNF-α and IL-1β in the serum, increased levels of TNF-α and greater microglial activation in the hippocampus. Additionally, correlation analysis revealed a positive association between the abundance of *Desulfobacterota* and the level of serum TNF-α in rats. Then, we performed abdominal surgery to investigate the impact of the preoperative gut microbiota on postoperative conditions, and the surgery did indeed cause POCD and inflammatory response. Notably, compared with rats that received preoperative fecal samples of control patients, rats that received preoperative fecal samples of POCD patients displayed exacerbated cognitive impairment; increased levels of TNF-α, IL-1β and IL-6 in the serum and hippocampus; and increased activation of microglia in the hippocampus.

*Correspondence:
Wei Zhang
doctor_anzw@163.com
Full list of author information is available at the end of the article

Conclusions Our findings suggest that the preoperative gut microbiota of POCD patients can induce preoperative and aggravate postoperative cognitive impairment and systemic inflammation in rats. Modulating inflammation by targeting the gut microbiota might be a promising approach for preventing POCD.

Keywords Preoperative gut microbiota, Postoperative cognitive dysfunction, Fecal microbiota transplantation, Neuroinflammation, Microglia

Introduction

The number of elderly patients undergoing surgery has significantly increased and is projected to increase further. Postoperative cognitive dysfunction (POCD), characterized by an objectively measurable decline in cognition at varying intervals, is common following surgery in elderly patients [1]. It is a complicated problem persisting well beyond the expected pharmacological and physiological effects of anesthetic drugs, leading to prolonged postoperative mechanical ventilation and hospitalization time, as well as increased morbidity and mortality [2].

There is a growing realization that the gut microbiota plays an essential role in regulating brain function through the gut-microbiota-brain axis and that gut microbiota dysbiosis is associated with many neurological disorders [3, 4]. Specifically, animal research has indicated that microbial dysbiosis is related to cognitive impairment and that probiotics have a beneficial effect on regulating cognitive function [5–7]. Clinically, although POCD occurs after surgery, it is related to preoperative indicators, including the gut microbiota. Notably, perioperative application of oral probiotics can decrease the incidence of POCD in elderly patients [8]. Additionally, typical features of the gut microbiota have been identified as relevant predictors for POCD [9, 10]. Therefore, it is essential to determine the impact of the preoperative gut microbiota on postoperative cognition to provide effective prevention strategies for POCD.

The communication between the gut and brain is mediated by multiple complex pathways, and signals from the gut microbiota may play important roles in the development of neuroinflammation-induced POCD [11, 12]. Nevertheless, microglial activation and proinflammatory cytokine secretion were also observed in patients with POCD [13]. While inhibiting neuroinflammation by regulating the gut microbiota can improve cognitive function [14]. Therefore, the aim of this study was to investigate the preoperative gut microbiota of elderly patients who underwent orthopedic surgery and to identify the potential of microbial biomarkers for the prediction of POCD. Moreover, fecal microbiota transplantation (FMT) experiments were conducted in rats to explore the impacts of preoperative gut microbiota from POCD patients on pre- and postoperative cognitive function and systemic inflammation.

Methods

Human donors and POCD assessment

This study was approved by the Ethics Committee for Scientific Research and Clinical Trials of the First Affiliated Hospital of Zhengzhou University (2022-KY-0919-001) and registered at https://www.chictr.org.cn/ (ChiCTR2200063571). Preoperative fecal samples were obtained from patients who underwent orthopedic surgery (prospective-specimen collection, retrospective-blinded-evaluation) at the First Affiliated Hospital of Zhengzhou University. All patients voluntarily participated in this study and signed an informed consent form. The inclusion criteria were as follows: (1) elderly (≥ 60 years old), (2) underwent orthopedic surgery (conventional open surgery—spine surgery or hip arthroplasty or knee surgery under general anesthesia), (3) had a body mass index (BMI) of 18–32 kg/m^2, (4) had an American Society of Anesthesiologists (ASA) physical status of I–II, and (5) had a preoperative Mini-Mental State Examination (MMSE) score based on different educational levels: score > 17 with 0–4 years of education, or score > 20 with 5–8 years of education, or score > 24 with more than 9 years of education [15]. The exclusion criteria were patients who (1) had a history of gastrointestinal surgery, (2) had digestive system diseases, or (3) had taken drugs that might have had an impact on the gut microbiota within one year. The elimination criteria were as follows: patients who (1) were unable to provide fecal samples before surgery, (2) changed the surgical approach to minimally invasive surgery, (3) suffered from serious complications during or after surgery, including massive bleeding, surgery time exceeding 4 h or ICU admission, or (4) refused postoperative follow-up.

Among the various neuropsychological tests, the MMSE is the most frequently used tool to identify POCD [16]. In this study, the participants underwent MMSE assessment by trained doctors at 2 time points: before surgery and within 7 days after surgery. After the preoperative interview with the MMSE, fecal samples were collected from eligible participants before surgery. All participants received standardized anesthesia and postoperative analgesia. General anesthesia was induced

sequentially with etomidate, alfentanil and rocuronium and was maintained with sevoflurane, propofol and remifentanil under the supervision of an experienced anesthesiologist according to individual clinical conditions. Postoperative pain was administered by acute pain services via a patient-controlled intravenous analgesia pump with 0.2 mg/kg hydromorphone in 200 mL of normal saline. POCD was defined as a decrease in the postoperative MMSE score of at least 3 points compared with the preoperative MMSE score [8, 17]. Participants who completed postoperative follow-up were classified into two groups: the POCD group and the control group, which were matched by sex, age (within 3 years of difference) and surgical type.

Animal recipients

Healthy adult male Sprague–Dawley rats (aged 18 months; body weight, 500 ± 20 g) were purchased from the Experimental Animal Center of Zhengzhou University. The animals were housed in an SPF-grade animal facility with free access to water and food. The facility had a 12 h light/dark cycle, a temperature of 20 ± 2 °C and a relative humidity of 40–60%. Rats were acclimatized to the new conditions for 2 weeks before FMT. Fifty rats were divided into 5 groups (n = 10): the C group, P group, Sham-C group, Surgery-C group, and Surgery-P group. C and P represented FMT with fecal microbiota from control patients and POCD patients, respectively. Sham and Surgery represented sham surgery and abdominal surgery, respectively. All animal experiments were approved by the Ethics Committee of the Experimental Animal Platform of the School of Medical Sciences, Zhengzhou University (ZZU-LAC2231201) and conducted according to the Guidelines for the Care and Use of Laboratory Animals.

Fecal microbiota transplantation (FMT)

Preoperative fecal samples from participants were collected into sterile tubes and frozen at − 80 °C until use. Control patients and POCD patients supplied different amounts of fecal samples and each sample was packaged after weighing. Before use, each sample was diluted with sterilized PBS buffer, and the mixture was vortexed, centrifuged and resuspended to 150 mg/ml of fecal suspension. One fecal suspension was used for each rat. As described in previous studies [18–20], rats were fed drinking water containing ampicillin (1 g/L), metronidazole (1 g/L), vancomycin (500 mg/L), ciprofloxacin hydrochloride (200 mg/L) and imipenem (250 mg/L) for 2 weeks to eliminate the indigenous gut microbiota. Then, FMT was performed thrice weekly for 3 weeks by oral gavage in a volume of 300 μL of homogenized fecal suspension. Rats in the C group, Sham-C group and Surgery-C group were transplanted with fecal microbiota from control patients. Rats in the P group and Surgery-P group were transplanted with fecal microbiota from POCD patients.

Surgery

Abdominal surgical procedures known to result in POCD were performed based on previous studies [21, 22]. Briefly, rats were anesthetized with 2–3% isoflurane for induction. Surgery was performed under 1.5–2% isoflurane anesthesia, and body temperature was maintained at 37 ± 0.5 °C. After the abdominal region was sterilized, a 3 cm vertical incision was made, and the abdominal cavity was exposed to explore the abdominal organs and musculature. Then, approximately 10 cm of the intestine was taken out and rubbed for 30 s using fingers. Next, the intestine was returned, and the abdominal wall along the incision was infiltrated with 0.25% ropivacaine to relieve surgery-related pain. Then, the surgical incision was closed from the peritoneal muscles to the skin by sterile suture, and the rats were placed on a heat blanket until anesthesia recovery. Rats in the Sham-C group were anesthetized and sterilized according to the above methods. Rats in the Surgery-C group and Surgery-P group received anesthesia and abdominal surgery according to the above methods.

Morris water maze (MWM)

The MWM test was performed to evaluate the learning and memory abilities of the rats. The test was conducted in a black circular tank (150 cm in diameter and 50 cm in height) filled with water (22 ± 2 °C) and divided into four quadrants. The escape platform (diameter 10 cm) was placed in the middle of one quadrant 1 cm below the water surface. The swimming path of each rat was recorded by an overhead video camera. The MWM test consisted of two sessions: the acquisition session and the probe trial session. During the acquisition session for 5 consecutive days, the rats were placed in the water facing the wall of the pool in one of the four quadrants. Each rat was allowed 120 s to find and mount the platform. When the rat found the platform, it was kept on the platform for 10 s. If the rat did not find the platform within 120 s, it was guided to the platform and allowed to stay on it for 10 s, after which the escape latency was recorded as 120 s. The escape latency, path length and swimming speed were recorded. On day 6 of the probe trial session, the escape platform was removed. Trained rats were placed in the quadrant opposite to the platform quadrant and allowed to swim for 120 s. The number of platform crossings and time spent in the targeted quadrant were recorded.

Enzyme-linked immunosorbent assay (ELISA)

The inflammatory cytokines of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) were measured using ELISA kits. For serum, blood samples were isolated by centrifugation and stored at − 80 °C until analysis. For the hippocampus, the samples were dissected, homogenized in RIPA lysis buffer and centrifuged. Subsequently, the supernatant was separated, and the total protein concentration was determined. Commercially available ELISA kits for detecting TNF-α (CER1393, CRK Pharma, Wuhan, China), IL-1β (CER1094, CRK Pharma, Wuhan, China) and IL-6 (CER0042, CRK Pharma, Wuhan, China) were used in accordance with the manufacturer's instructions.

Immunofluorescence

The expression of ionized calcium binding adaptor molecule 1 (Iba-1) was detected by immunofluorescence staining to observe the microglia in the hippocampus. The brain sections were immersed, deparaffinized, hydrated and boiled for antigen retrieval. After washing with PBS, the sections were incubated with 3% bovine serum albumin (BSA)/10% normal goat serum for 30 min to block nonspecific binding. Then, the sections were incubated in sequence with primary anti-Iba-1 antibody (ab178846, Abcam) at 4 °C overnight and the secondary antibody goat anti-rabbit IgG-H&L (Alexa Fluor® 647) (ab150079, 1:1000, Abcam) at room temperature in the dark. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Fluorescence signals were obtained by laser scanning confocal microscopy (Nikon, Tokyo, Japan).

16S rRNA sequencing analysis

For 16S rRNA sequencing analysis, DNA was extracted using E.Z.N.A. ® Stool DNA Kit (Omega Bio-tek, Inc., GA, USA). The V3-V4 region of the bacterial 16S rRNA gene was amplified with the universal primers F1 and R2 (5′-CCTACGGGNGGCWGCAG-3′ and 5′-GACTAC HVGGGTATCTAATC-C-3′). Subsequently, PCRs were run in a T100™ Thermal Cycler PCR system (Bio-Rad Laboratories, Inc., CA, USA). The products were purified and then sequenced using the MiSeq platform (Illumina Inc., CA, USA). The bacterial communities were calculated using amplicon sequence variants (ASVs), and the diversity was analyzed by QIIME2 and R software (v3.6.1). The α-diversity was calculated using the abundance-based coverage estimator (ACE), Chao, Shannon and Simpson indices. The β-diversity was examined using Principal Coordinates Analysis (PCoA) based on weighted UniFrac distances with visualization of the grouping [23, 24]. The statistical comparison of the relative abundances of bacterial communities was conducted using the Wilcoxon rank sum test. Linear discriminant analysis Effect Size (LEfSe) was used to analyze the crucial bacterial communities from the phylum to genus level. PICRUSt2 software was used to align the functional gene sequences with the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database.

Statistical analysis

Statistical analysis in this study was conducted using SPSS 22.0 software (IBM Corp., Armonk, NY, USA) and GraphPad Prism 9 (GraphPad Software, San Diego, CA). Continuous variables between the two groups with normal distribution were analyzed using *t* test after a homogeneity of variance test; otherwise, the Wilcoxon rank sum test was used. Categorical variables were presented as n (%) and were compared with the Wilcoxon test or Fisher's exact test. The associations between clinical variables and bacterial communities were tested by multivariable association with linear models 2 (MaAsLin2). Logistic regression was used to establish and compare the predictive models. Escape latency was compared by repeated-measures ANOVA. Correlations between the abundance of microbial communities and inflammatory cytokine levels were analyzed by Spearman rank correlation. Statistical comparisons of three groups were conducted using one-way ANOVA or the Kruskal–Wallis test. $P < 0.05$ was considered statistically significant.

Results

POCD patients exhibit a shift in the preoperative gut microbiota composition

In the present study, 125 patients provided qualified preoperative fecal samples and were successfully followed up (Additional file 1: Table S1). Among them, 24 patients were included in the POCD group, and the incidence was 19.2%. Another 24 patients were matched by sex, age and surgical type and were included in the control group. The clinical characteristics of the 48 patients included in the microbial analysis were shown in Additional file 1: Table S2. There were no significant differences in education level, preoperative ESR, preoperative CRP, operative time, recovery time or VAS pain score between the two groups. However, the BMI in the POCD group was higher than that in the control group (25.3 ± 2.8 vs 23.7 ± 2.5) (Fig. 1A). In addition, the preoperative MMSE score in the POCD group was significantly lower than that in the control group (22.7 ± 4.0 vs 26.7 ± 2.9) (Fig. 1B).

Then, we analyzed the gut microbiota in preoperative fecal samples from the 48 participants in the two groups by 16S rRNA gene sequencing analysis. As shown in the Venn diagram (Fig. 1C), a total of 933 ASVs were detected. There were 577 overlapping ASVs between the two groups, 169 belonging to the POCD

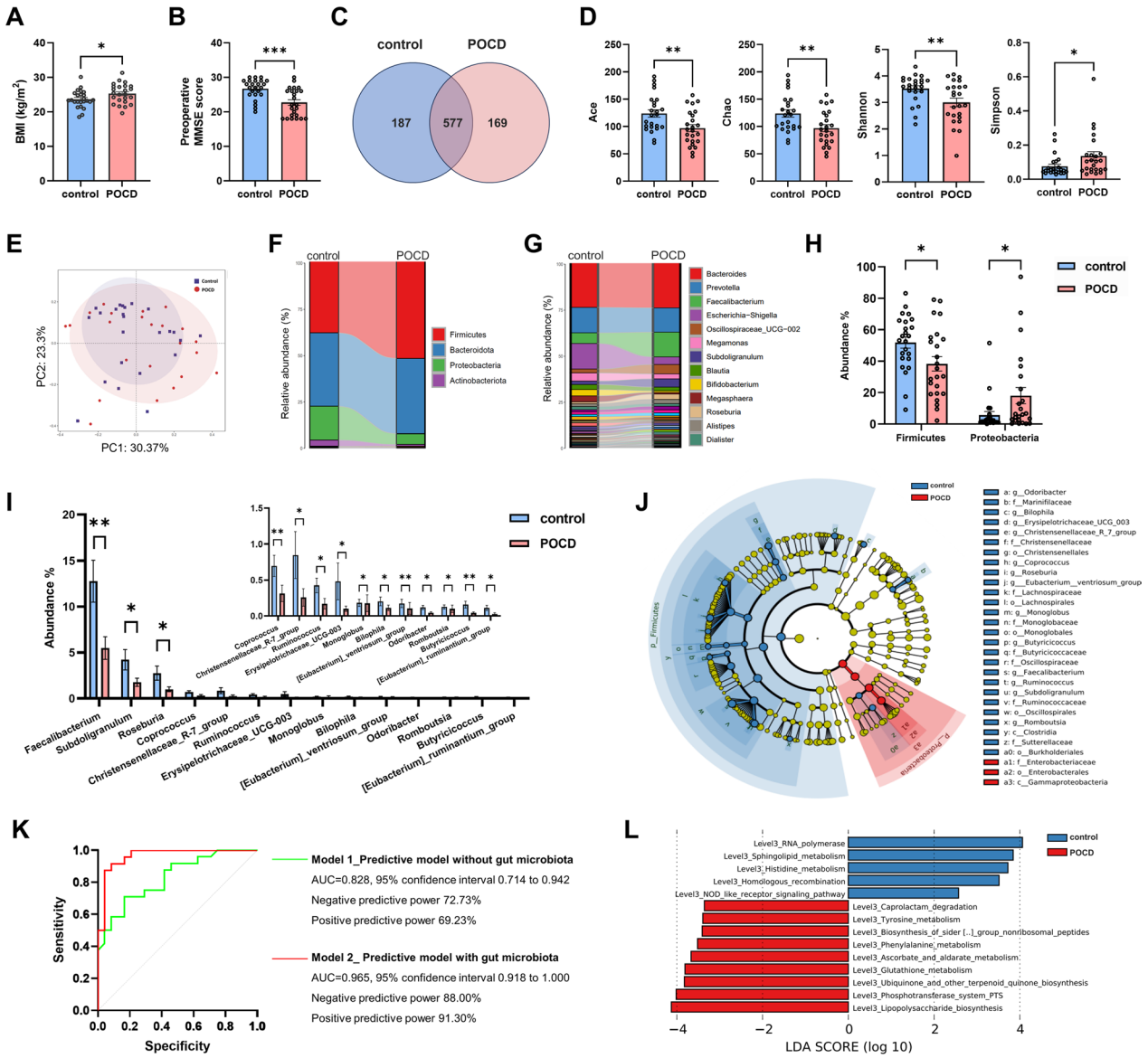

Fig. 1 POCD patients exhibit a shift in the preoperative gut microbiota composition. Comparison of BMI (**A**) and preoperative MMSE score (**B**) between POCD patients (n = 24) and control patients (n = 24). **C** Venn diagram of ASVs. **D** The α-diversity indices of the ACE, Chao, Shannon and Simpson indices. **E** The β-diversity analysis of the PCoA plot based on weighted UniFrac distances. Changes in the relative abundances of the main bacterial communities at the phylum (**F**) and genus (**G**) levels. The relative abundances of bacterial communities with significant differences at the phylum (**H**) and genus (**I**) levels. **J** Cladogram of LEfSe results comparing bacterial communities from the phylum to genus level. **K** ROC curves of the two predictive models with or without data on the preoperative gut microbiota. **L** KEGG pathway functional gene difference analysis. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

group and 187 belonging to the control group. According to the α-diversity analysis [23], the POCD group had significantly higher ACE, Chao and Shannon indices (the higher the value, the higher the richness), but lower Simpson indices (the higher the value, the lower the richness) than the control group (Fig. 1D), suggesting that POCD patients had lower bacterial richness. For β-diversity analysis [24], PCoA analysis based on weighted UniFrac distances revealed that the POCD group had a relatively larger distribution area than the control group (Fig. 1E). Changes in the main bacterial communities at the phylum and genus levels were shown in Fig. 1F, G. At the phylum level, the relative abundance of *Firmicutes* significantly decreased, while that of *Proteobacteria* significantly increased in the POCD group (Fig. 1H). There was no statistically significant difference

in the *Firmicutes/Bacteroidetes* ratio (F/B ratio) between the two groups (Additional file 1: Fig. S1A). The communities with significant differences between the two groups at the genus level were shown in Fig. 1I. LEfSe cladogram analysis revealed crucial bacterial communities from the phylum to genus level between the two groups (Fig. 1J).

To identify the crucial bacterial communities that were not affected by clinical variables, we tested the relationships between the preoperative clinical variables (BMI, education level, preoperative MMSE score, ESR and CRP) and differential microbial communities by MaAsLin2. The parameter q value < 0.25 was considered statistically significant and we did not find any statistically significant associations. Therefore, the crucial bacterial communities identified by LEfSe were all related to POCD grouping. Next, we explored the predictive potential of the crucial bacterial communities. According to the logistic regression results, preoperative clinical indicators of BMI and preoperative MMSE score were selected to construct a predictive model (Model 1). The model had an area under the ROC curve (AUC) of 0.828. Among the crucial bacterial communities, the order *Oscillospirales* and three families, *Ruminococcaceae, Oscillospiraceae* and *Butyricicoccaceae,* were selected to construct a predictive model combined with BMI and preoperative MMSE score (Model 2). This model had an AUC of 0.965, suggesting the potential of preoperative crucial bacterial communities to predict POCD (Fig. 1K). In addition, KEGG pathway analysis revealed significant functional gene differences, as shown in Fig. 1L. Taken together, these findings indicated that POCD patients exhibit a shift in the preoperative gut microbiota composition.

Rats that received FMT from preoperative fecal samples of POCD patients exhibit a shift in gut microbiota composition

To study the impact of the preoperative gut microbiota of POCD patients on brain health, rats in the C group (n = 10) and P group (n = 10) were transplanted with fecal microbiota from either control patients or POCD patients, respectively. After the MWM test, fecal samples were collected from the rats in sterile tubes and frozen at − 80 °C until analysis (Fig. 2A). The fecal samples were analyzed by 16S rRNA gene sequencing analysis, which revealed no significant differences in α-diversity or β-diversity between the two groups. As

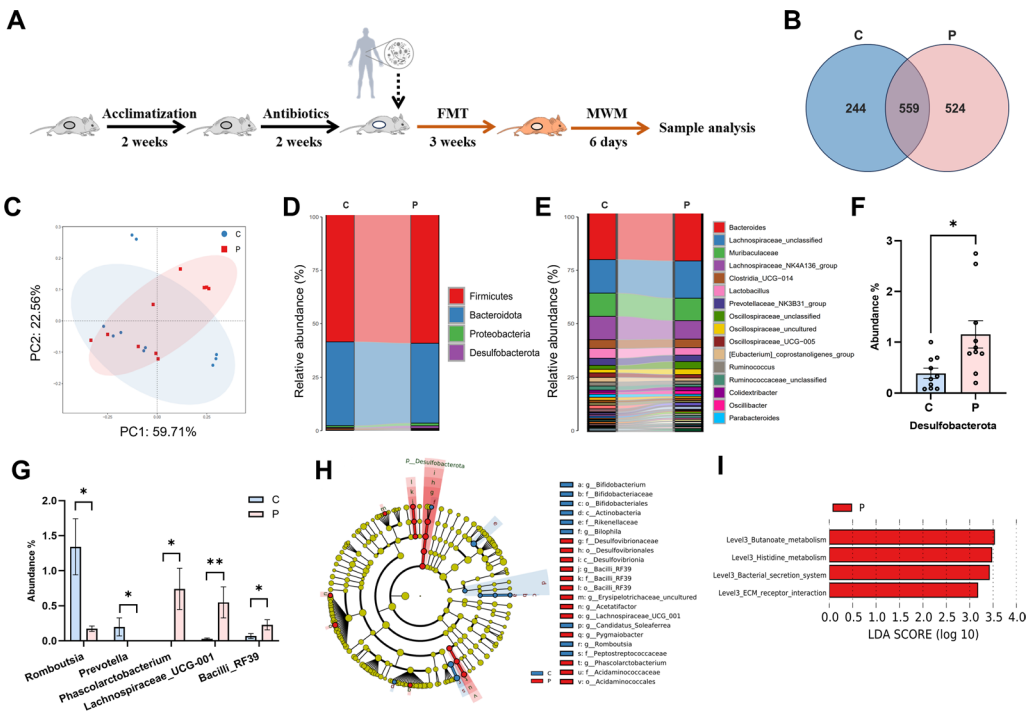

Fig. 2 Rats that received FMT exhibit a shift in gut microbiota composition. **A** Experimental design. After 2 weeks of acclimatization, the rats were treated with antibiotics for 2 weeks. Then, fecal suspensions from either control patients or POCD patients were transferred to C group (n = 10) or P group (n = 10) rats for 3 weeks. After 6 days of the MWM test, fecal samples, blood samples and brain tissue were collected from the rats for further analysis. **B** Venn diagram of ASVs. **C** PCoA plot based on weighted UniFrac distances between the two groups. Changes in the relative abundances of the main bacterial communities at the phylum (**D**) and genus (**E**) levels. **F** The relative abundance of the *Desulfobacterota* phylum. **G** The bacterial communities with significant differences at genus levels. **H** Cladogram of LEfSe results comparing bacterial communities from the phylum to genus level. **I** KEGG pathway functional gene difference analysis. Data are expressed as mean ± SEM. **P* < 0.05, ***P* < 0.01

shown in the Venn diagram (Fig. 2B), a total of 1327 ASVs were detected. There were 559 overlapping ASVs between the two groups, 524 belonging to the P group and 244 belonging to the C group. The PCoA plot based on weighted UniFrac distances was shown in Fig. 2C. Changes in the main bacterial communities at the phylum and genus levels were shown in Fig. 2D, E. Compared with that in the C group, the abundance of the *Desulfobacterota* phylum significantly increased in the P group (Fig. 2F). The F/B ratios of the two groups were not significantly different (Additional file 1: Fig. S1B). The communities with significant differences between the two groups at the genus level were shown in Fig. 2G. The cladogram of LEfSe analysis showed crucial bacterial communities from the phylum to genus level between the two groups (Fig. 2H). In addition, KEGG pathway analysis revealed four significantly different functional gene pathways whose expression increased in the P group (Fig. 2G).

Rats that received FMT from preoperative fecal samples of POCD patients exhibit cognitive impairment and systemic inflammation

After FMT, the MWM test was conducted to measure cognitive function (Fig. 2A). In the acquisition session, the escape latency of the rats in both groups tended to decrease, while the C group rats spent less time finding the target platform from day 3 to day 5, suggesting that the P group rats exhibited worse spatial learning performance (Fig. 3A). No motor dysfunction was detected according to the swimming speed results (Fig. 3B). In the probe trial session, rats in the P group spent less time (%) in the target quadrant (Fig. 3C) and crossed the platform less frequently than did those in the C group (Fig. 3D).

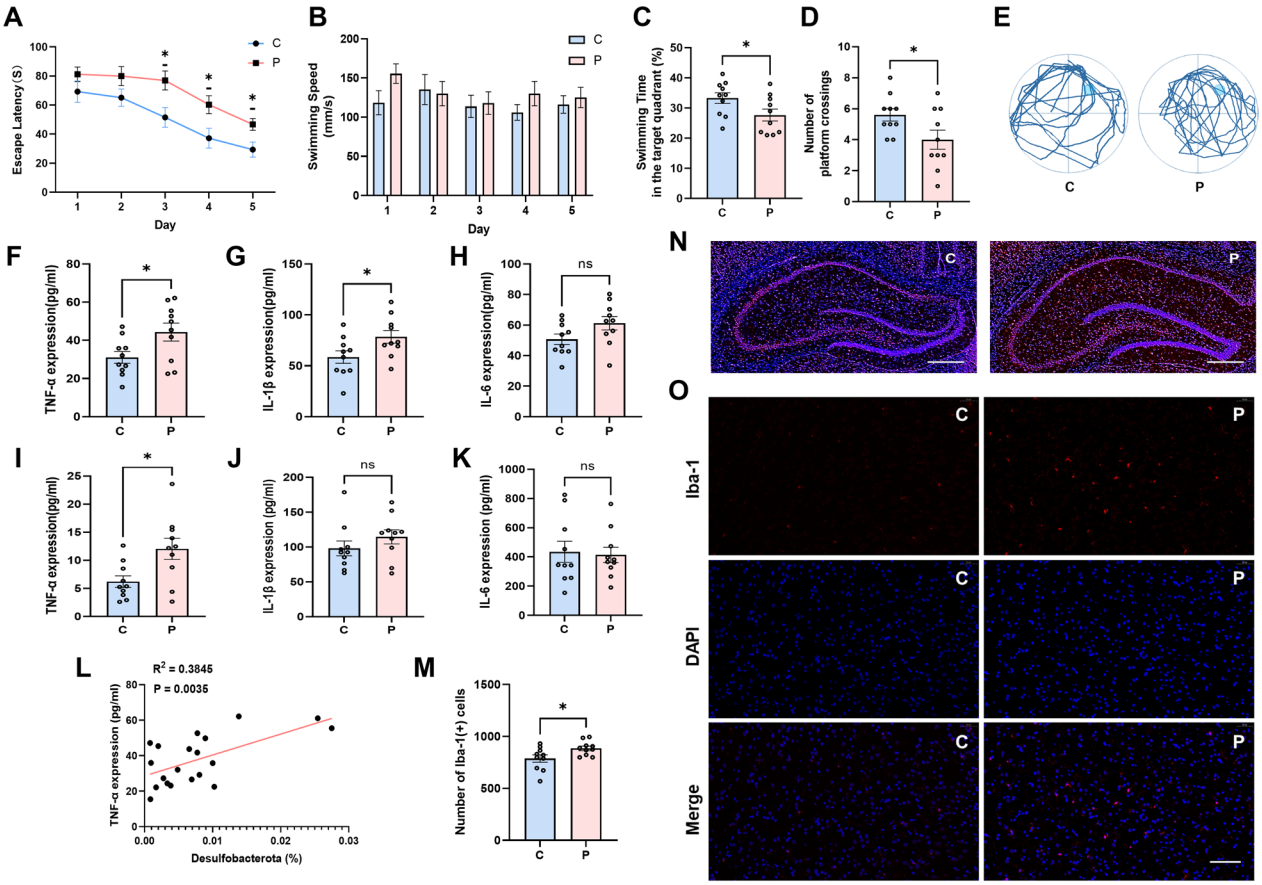

Fig. 3 Rats that received FMT exhibit cognitive impairment and systemic inflammation. Escape latency (**A**) and swimming speed (**B**) of the C group and P group rats from days 1 to 5 during the acquisition session of the MWM test. The swimming time in the target quadrant (**C**) and number of platform crossings (**D**) of the C group and P group rats on day 6 during the probe trial session of the MWM test**. E** Typical motion traces of the rats in the MWM test. Levels of TNF-α (**F**), IL-1β (**G**) and IL-6 (**H**) in the serum of the rats. Levels of TNF-α (**I**), IL-1β (**J**) and IL-6 (**K**) in the hippocampus of the rats. **L** Spearman rank correlation analysis of the relative abundance of the *Desulfobacterota* phylum and the level of TNF-α in the serum of the rats. **M** Quantitative analysis of Iba-1$^+$ (microglial marker) cells. **N** Representative immunofluorescence images of Iba-1$^+$ (red) staining in the hippocampus of rats (scale bar = 500 μm). **O** Representative immunofluorescence images of nuclei (blue) and Iba-1$^+$ (red) staining in the hippocampus of rats (scale bar = 100 μm). Data are expressed as mean ± SEM. $^*P < 0.05$, $^{ns}P \geq 0.05$

Figure 3E illustrated the typical motion traces during the MWM test. The results indicated that the preoperative gut microbiota of POCD patients could induce cognitive impairment in rats.

After the MWM test, rats were anesthetized with isoflurane and sacrificed by perfusion with normal saline. Blood samples and brain tissue were harvested (Fig. 2A). To investigate the impact of the preoperative gut microbiota of POCD patients on peripheral inflammation in rats, we detected the levels of the inflammatory cytokines TNF-α, IL-1β and IL-6 in the serum by ELISA. Compared with those in the C group, TNF-α and IL-1β expression was significantly increased in the P group (Fig. 3F, G). However, IL-6 expression did not significantly differ between the two groups (Fig. 3H). We also detected inflammatory cytokines in the hippocampus and found that TNF-α expression was significantly increased in the P group than in the C group (Fig. 3I). However, IL-1β and IL-6 expression did not significantly differ between the two groups (Fig. 3J, K). We further conducted a correlation analysis of the inflammatory cytokines and microbial communities. Interestingly, the relative abundance of the *Desulfobacterota* phylum showed a positive association with the level of TNF-α in the serum (Fig. 3L), suggesting the impact of microbial dysbiosis on peripheral inflammation. Then, activated microglia in the hippocampus were observed by Iba-1 staining. Compared with that in the C group, the Iba-1^+ cells were significantly increased in the P group (Fig. 3M), suggesting a neuroinflammatory response. Representative immunofluorescence images were shown in Fig. 3N, O. Taken together, the results indicated that the preoperative gut microbiota of POCD patients could induce neuroinflammation in rats and the peripheral inflammation might be one of the potential mechanisms.

Changes in the preoperative gut microbiota aggravate postoperative cognitive impairment and systemic inflammation in rats

To investigate the impact of the preoperative gut microbiota of POCD patients on postoperative cognitive function and inflammatory response in rats, FMT experiment was repeated in 30 rats that were randomly divided into 3 groups, namely, the Sham-C group (n = 10) and Surgery-C group (n = 10) rats, which received fecal microbiota from control patients, and the Surgery-P group (n = 10) rats, which received fecal microbiota from POCD patients. Rats in the Surgery-C and Surgery-P groups underwent abdominal surgery to establish the POCD model, and rats in the Sham-C group underwent sham surgery. Next, the MWM test was conducted on postoperative days 7–12, and the serum and hippocampal samples were analyzed (Fig. 4A).

In the acquisition session, the escape latency of the rats in each group tended to decrease. Compared with Surgery-C group rats, Sham-C group rats required less time to find the target platform from day 4 to day 5, and Surgery-P group rats required more time to find the target platform from day 2 to day 5, suggesting that Surgery-P group rats exhibited the worst spatial learning performance (Fig. 4B). No motor dysfunction was detected according to swimming speed (Fig. 4C). In the probe trial session, the Sham-C group rats spent less time (%) in the target quadrant and crossed the platform less frequently, and the Surgery-P group rats spent more time (%) in the target quadrant and crossed the platform more frequently than did the Surgery-C group rats (Fig. 4D, E), indicating the aggravated cognitive impairment caused by the surgical trauma and microbial dysbiosis. Typical motion traces of the three groups were shown in Fig. 4F.

Additionally, the inflammatory cytokines in the serum and hippocampus of the rats were detected by ELISA. The results showed that, both in the serum (Fig. 4G–I) and in the hippocampus (Fig. 4J–L), the TNF-α, IL-1β and IL-6 levels were significantly lower in the Sham-C group and higher in the Surgery-P group compared with that in the Surgery-C group, indicating the surgery-induced inflammatory response, as well as the greater secretion of proinflammatory cytokines mediated by the microbial dysbiosis. Then, Iba-1 staining of the hippocampus was performed to observe the activated microglia, and representative immunofluorescence images were shown in Fig. 4M, N. Compared with that in the Surgery-C group, the Iba-1^+ cells were significantly decreased in the Sham-C group and increased in the Surgery-P group (Fig. 4O), suggesting the aggravated neuroinflammation caused by the surgical trauma as well as microbial dysbiosis. Taken together, these results indicated that the changes in preoperative gut microbiota from POCD patients could aggravate POCD and systemic inflammation in rats.

Discussion

In this study, we investigated the preoperative gut microbiota of POCD patients by 16S rRNA gene sequencing analysis and demonstrated the differences in the characteristics and the predictive potential for POCD patients. Furthermore, we investigated the impact of the preoperative gut microbiota from POCD patients on cognitive function and systemic inflammation in rats by FMT, suggesting aggravated cognitive impairment and inflammatory responses from the pre- to postoperative stages (Fig. 5).

In clinical researches, the inconsistent diagnosis of POCD has led to significant variation between published studies, and the incidence of POCD varies from 10 to 40% at 7 days after different types of surgeries [25]. In

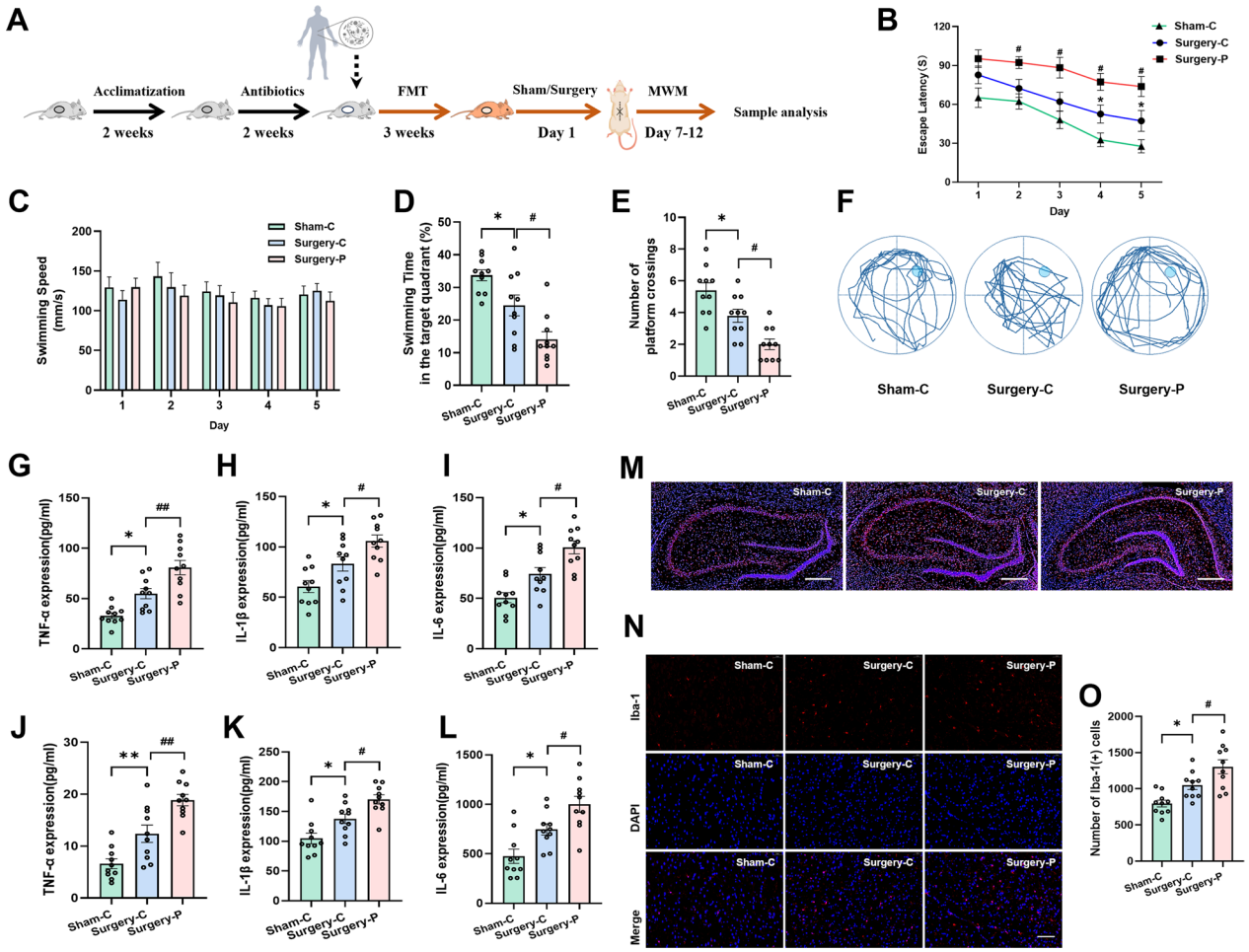

Fig. 4 Changes in the preoperative gut microbiota aggravate POCD and systemic inflammation in rats. **A** Experimental design. After 2 weeks of acclimatization and 2 weeks of antibiotic treatment, fecal material from either control patients or POCD patients was transferred to Sham-C group (n = 10)/Surgery-C group (n = 10) or Surgery-P group (n = 10) rats for 3 weeks. Then, the rats underwent sham surgery or abdominal surgery. The MWM test was conducted on postoperative days 7–12. Serum and hippocampal samples were subsequently analyzed. Escape latency (**B**) and swimming speed (**C**) of the three groups of rats from days 1 to 5 during the acquisition session of MWM test. The swimming time in the target quadrant (**D**) and number of platform crossings (**E**) of the three groups of rats on day 6 during the probe trial session of the MWM test. **F** Typical motion traces of the three groups of rats in the MWM test. Levels of TNF-α (**G**), IL-1β (**H**) and IL-6 (**I**) in the serum of rats. Levels of TNF-α (**J**), IL-1β (**K**) and IL-6 (**L**) in the hippocampus of rats. **M** Representative immunofluorescence images of Iba-1$^+$ (red) staining in the hippocampus of rats (scale bar = 500 μm). **N** Representative immunofluorescence images of nuclei (blue) and Iba-1$^+$ (red) staining in the hippocampus of rats (scale bar = 100 μm). **O** Quantitative analysis of Iba-1$^+$ (microglial marker) cells. Data are expressed as mean ± SEM. compared with Sham-C group, $^*P < 0.05$, $^{**}P < 0.01$; compared with Surgery-C group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$

this study, the incidence of POCD was 19.2% in elderly patients who underwent orthopedic surgery, which was similar to the median incidence of 19.3% reported in previous studies [26]. Several risk factors for POCD have been characterized, such as old age, preoperative inflammation, long operative time and postoperative pain [27]. In this study, the POCD patients had higher BMI and lower preoperative MMSE score. On the one hand, many studies have shown the association between high BMI and cognitive dysfunction in elderly patients [28, 29]. In addition, the increased F/B ratio has been suggested to be related to obesity [30, 31], whereas we found no significant difference in the F/B ratio between the control and POCD patients. On the other hand, the lower preoperative MMSE score in the POCD group indicated that preoperative mild cognitive impairment that was not recognized by the MMSE could increase the risk of POCD, which was consistent with the findings of previous studies [32, 33].

The predictive potential of the preoperative gut microbiota for POCD has been revealed in previous studies [9]. Notably, preoperative stress can alter the gut

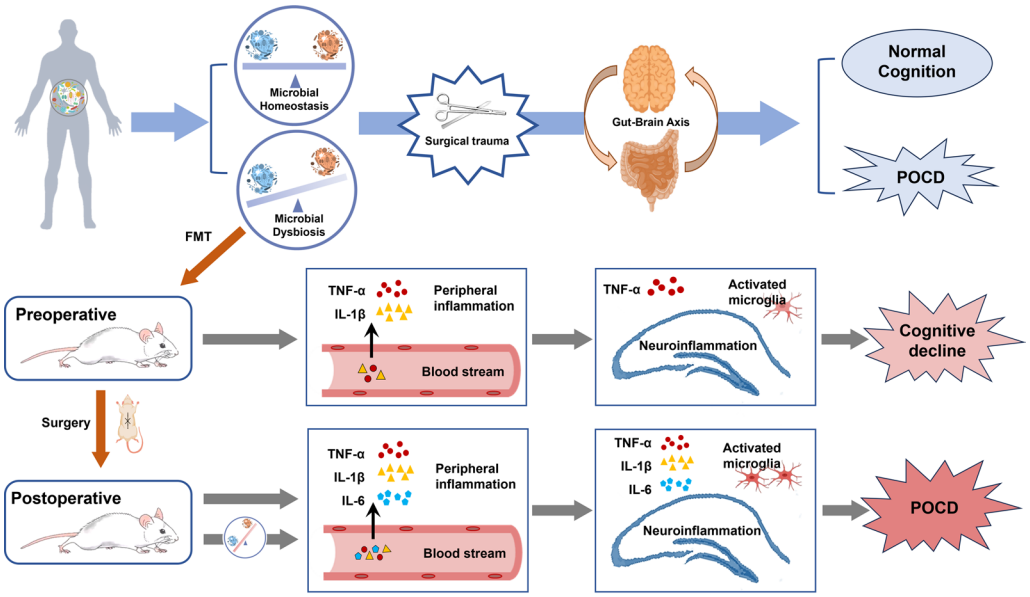

Fig. 5 Schematic illustration of the impact of the preoperative gut microbiota on POCD. Dysbiosis of the preoperative gut microbiota, with an increase in proinflammatory bacteria and a decrease in anti-inflammatory bacteria, is associated with POCD via regulation of the gut-microbiota-brain axis. In rats receiving FMT from the preoperative gut microbiota of POCD patients, dysbiosis of the gut microbiota induces preoperative and aggravates postoperative peripheral inflammation, causing varying degrees of neuroinflammation and cognitive impairment

microbiota and aggravate postoperative emotional deficits [34]. Additionally, probiotic therapy can improve postoperative cognitive function [8]. However, due to differences in diet and lifestyle in different regions, the composition of microbial communities also varies. Overall, microbial dysbiosis in patients with cognitive impairment is characterized by an increase in proinflammatory bacteria and a decrease in anti-inflammatory bacteria [35]. In this study, *Proteobacteria*, which is one of the most abundant phyla in humans and contains several opportunistic pathogens, exhibited increased abundance in POCD patients. This increase has been demonstrated in several neurodegenerative diseases and cognitive deficits [36–38]. Additionally, *Firmicutes*, which is the predominant bacterial phylum and has beneficial effects on health, had a decreased abundance in POCD patients. Similarly, its decrease has been observed in Parkinson's disease patients [39, 40].

Nevertheless, the mechanisms by which the preoperative gut microbiota affects brain function remain to be elucidated. In the present study, we used FMT to investigate the shift in microbial composition in rats. We found the abundance of the *Desulfobacterota* phylum, which has been found to be related to various neurological diseases, significantly increased in the P group rats. Besides, in the stress-induced depressive-like behavior model of rats, there was also an increase of *Desulfobacterota*, and the depressive-like behavior could be improved by reducing the *Desulfobacterota* [41]. Additionally, in mouse model of Parkinson's disease, the motor impairments and neuronal deficits could be alleviated by suppressing *Desulfobacterota* [42]. However, although it is commonly used, FMT lacks methodological standardization, and its influential factors should be considered when designing studies [18]. A comparative evaluation study suggested that the recipient rats could establish a bacterial community more similar to human-donor than the recipient mice [43]. Therefore, rats were selected in this study. In addition, the antibiotic-depleted microbiota model was conducted based on previous studies [19, 20, 44–46], but it could not completely eliminate the indigenous gut microbiota.

Along with the shift in the gut microbiota, the rats receiving fecal samples from POCD patients exhibited increased TNF-α levels in the serum and hippocampus. TNF-α is a proinflammatory cytokine predominantly secreted by macrophages. It is involved in the regulation of inflammation and neurological disorders including POCD [47]. In the hippocampus, increased levels of TNF-α also indicate microglial activation [48, 49]. Previous evidence has shown that inhibiting TNF-α can reduce neuroinflammation and alleviate POCD in aged rats [50]. Furthermore, our findings revealed a correlation between the TNF-α level in serum and the relative

abundance of the *Desulfobacterota* phylum, indicating that the inflammation plays an essential role in the gut-microbiota-brain axis [51, 52].

The results of the FMT experiment in this study could only explain the preoperative situation. Therefore, we further used the POCD model to explore the impact of the preoperative gut microbiota on the postoperative outcome. The comparison between the Sham-C group and Surgery-C group indicated successful establishment of the POCD model. Notably, the comparison between the Surgery-C group and Surgery-P group indicated aggravated cognitive impairment and neuroinflammation mediated by the preoperative gut microbiota. Indeed, POCD is a multifactorial disorder. Systemic inflammation caused by surgery plays a significant role in the pathogenesis of POCD [48, 52]. Not only can the gut microbiota directly control the neuroinflammation, but surgery can also affect neuroinflammation through the gut microbiota [14, 53]. Given that, we speculate two possible mechanisms by which POCD is induced by the preoperative gut microbiota. One is that preoperative neuroinflammation (induced by the preoperative gut microbiota) is directly aggravated by surgery. The other is that surgery causes microbial dysbiosis, aggravating postoperative neuroinflammation through the gut-microbiota-brain axis (Fig. 5).

Specifically, the IL-1β in the hippocampus only increased after surgery and the reason might be related to its paradoxical effect on the central nervous system (CNS). IL-1β is an inducible cytokine predominantly secreted by blood myeloid cells, lymphocytes, CNS microglia and astrocytes [54]. In Alzheimer's disease, IL-1β in the CNS not only participates in proinflammatory responses but also regulates the physiology of microglia by upregulating the expression of anti-inflammatory mediators [55]. Moreover, the IL-6 in the serum and hippocampus only increased after surgery and the reason might be related to its dynamic changes [56]. Clinical study has shown its dynamic changes in plasma which is associated with postoperative cognitive function [57]. In addition, different animals, inflammatory cytokines, brain regions, etc., may also have different results in different experiments [58, 59].

Several limitations in this study should be noted. First, the sample size of the recruited patients for microbiota analysis is too small. Second, the assessment of POCD is based on the MMSE, which is insufficient in screening mild cognitive dysfunction [60]. Third, in the FMT experiment, the exploration of the mechanisms involved only inflammation, without in-depth research on the intestinal barrier, blood–brain barrier or metabolism. Fourth, due to the difficulty in obtaining qualified postoperative fecal samples, we did not study the postoperative gut microbiota, which should be considered in future research. Moreover, we cannot exclude the possibility that our results were affected by an uncontrolled confounder.

Conclusions

In summary, our study suggests that POCD patients exhibit a shift in the preoperative gut microbiota. Through FMT, we provide evidence that the preoperative gut microbiota of POCD patients can induce peripheral inflammation, neuroinflammation and microglial activation, leading to cognitive impairment in rats. In addition, mediated by the dysbiosis of preoperative gut microbiota, surgical trauma can aggravate postoperative cognitive impairment and neuroinflammation. Therefore, targeting the gut microbiota to regulate inflammation may be a promising strategy for preventing POCD.

Abbreviations

POCD	Postoperative cognitive dysfunction
FMT	Fecal microbiota transplantation
BMI	Body mass index
ASA	American Society of Anesthesiologists
MMSE	Mini-Mental State Examination
MWM	Morris water maze
ELISA	Enzyme-linked immunosorbent assay
TNF-α	Tumor necrosis factor-α
IL-1β	Interleukin-1β
IL-6	Interleukin-6
Iba-1	Ionized calcium binding adaptor molecule 1
ASV	Amplicon sequence variant
ACE	Abundance-based coverage estimator
PCoA	Principal Coordinates Analysis
LEfSe	Linear discriminant analysis effect size
KEGG	Kyoto Encyclopedia of Genes and Genomes
MaAsLin	Multivariable association with linear models
AUC	Area under the ROC curve
CNS	Central nervous system

Supplementary Information

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Additional file 1: Table S1. Clinical characteristics of the 125 patients. **Table S2.** Clinical characteristics of the 48 patients. **Figure S1.** Firmicutes/Bacteroidetes ratio (F/B ratio) of the gut microbiota.

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Author contributions

XW, FX and WZ contributed to the study conception and design. XW, FZ, DC, YHZ and FZ contributed to clinical data collection, sample collection and neurological assessments. XW, FX, YWX and FZ contributed to the animal experiments. YWX, FZ, DC and YHZ contributed to the ELISA and immunofluorescence experiments. XW, FX and WZ contributed to the data analysis

and manuscript writing. All the authors have read and approved the final manuscript.

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Availability of data and materials
All the data used and/or analyzed in this study are presented in the main text and additional files. Additional data and materials are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
The clinical study was approved by the Ethics Committee for Scientific Research and Clinical Trials of the First Affiliated Hospital of Zhengzhou University (2022-KY-0919–001) and registered at https://www.chictr.org.cn/ (ChiCTR2200063571). All participants provided written informed consent. The animal experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals and approved by the Ethics Committee of the Experimental Animal Platform of the School of Medical Sciences, Zhengzhou University (ZZU-LAC2231201).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Department of Anesthesiology, Pain and Perioperative Medicine, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China. [2]Department of Orthopedics, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China.

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