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NF-κB/P65 signaling pathway: a potential therapeutic target in postoperative cognitive dysfunction after sevoflurane anesthesia

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Abstract. – OBJECTIVE: This study aimed to explore the role of NF-κB/P65 signaling pathway in postoperative cognitive dysfunction (POCD) after sevoflurane anesthesia.

MATERIALS AND METHODS: A total of 120 male Sprague-Dawley (SD) rats were selected and assigned into five groups (24 rats in each group): the control, sevoflurane, sevoflurane + splenectomy, pyrrolidine dithiocarbamate (PDTC, a specific inhibitor of NF-κB), and sevoflurane + splenectomy + PDTC groups. Electrocardiogram (ECoG) and behavior changes of rats were monitored before and after anesthesia/operation. Ionized calcium-binding adapter molecules 1 (Iba-1) in the hippocampal zones were observed by immunofluorescence staining. Blood-brain barrier (BBB) permeability was determined by immunohistochemistry. The mRNA and protein expressions of NF-κB/P65 signaling pathway-related proteins and inflammatory cytokines were detected by qRT-PCR assay and Western blotting.

RESULTS: During the anesthesia/operation, the vital signs of rats were stable, but the ECoG in the sevoflurane and sevoflurane + splenectomy groups mainly presented slow waves. The ECoG arousal response in the sevoflurane + splenectomy + PDTC group was observed. At 24 h after the anesthesia/operation, the expressions of NF-κB and P65 in the hippocampal zone, the expressions of IκBα and inflammatory cytokines (IL-1β, IL-6 and TNF-α), the expression of Iba-1 in rat hippocampal dentate gyrus (DG) zone and CA3 zone, and the permeability of BBB were significantly increased and the behavior of rats changed dramatically (all $p < 0.05$), while PDTC treatments could eliminate these changes induced by the anesthesia/operation (all $p < 0.05$). No changes were observed in the expressions of NF-κB, P65, IκBα, Iba-1 and inflammatory cytokines (IL-1β, IL-6 and TNF-α), and the permeability of BBB and the behavior of rats in the sevoflurane and the PDTC groups (all $p > 0.05$).

CONCLUSIONS: These results suggest that the inhibition of NF-κB/P65 signaling pathway may relieve POCD after sevoflurane anesthesia.

Key Words:
NF-κB/P65 signaling pathway, Sevoflurane, Anesthesia, Cognitive dysfunction.

Introduction

Cognitive dysfunction, a disease in the brain organs, often causes functional disorders in a patient's perception, memory, attention and other brain functions[1]. Cognitive disorders include agnosia, deficiencies in processing speed, movement dysfunction, apraxia, attention disorders and memory loss[2]. Mild cognitive impairments affect memory and attention ability, while severe impairments cause dementia and even vegetative conditions[3]. According to a research report[4], people with the mild cognitive impairment are at increased risk of dementia, although the conversion rate is 21.9% over a period of 3 years. Moreover, substantial and persistent new cognitive impairment is reported to be independently associated with severe sepsis[5]. Therefore, cognitive impairment poses a serious threat, and hence it is important to explore effective molecular for the diagnosis and treatment of cognitive impairments.

Nuclear factor kappa B (NF-κB) is a nuclear transcription factor that participated in the control of a variety of cellular processes, and the activation of NF-κB is important for the cellular stress response to several factors (cytokine stimulation and irradiation)[6]. Some neurotrophic factors can activate NF-κB/P65 and induce the activation of genes, which are related to neural cell activation, differentiation, survival and growth, such as the inhibition of apoptosis factor Bcl-2 and the induction of apoptotic factor Bax[7]. Also, NF-κB is an inducing factor for the inflammatory response in glial cells, whose target genes include proinflammatory cytokines, such as IL-6 and TNF-α[8,9]. According to relevant research[10],

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inflammation plays a regulatory role in the pathology of brain injury. Therefore, the expressions of NF-κB and P65 may be important during the regulation of cognitive dysfunction. Additionally, surgical induced cognitive impairment is one of the main causes of cognitive impairments, for the anesthesia used during the operation damages the orientation, memory, thinking and other abilities of patients[11,12]. It is proved that the use of sevoflurane often causes transient postoperative cognitive dysfunction (POCD)[13]. Therefore, it is speculated that POCD may be related to the activation of inflammatory factors or pro-inflammatory factors under operation and anesthesia. On this regards, this study aims to investigate the role of NF-κB/P65 signaling pathway in POCD after sevoflurane anesthesia.

Materials and Methods

Ethic Statement

The present work performed in agreement with the approved animal protocols and guidelines established by Medicine Ethics Review Committee for Animal Experiments to protect the care and use of laboratory animals. All experiments were in strict accordance with relevant regulations set by the International Association for the Study of Pain for the protection and use of laboratory animals[14].

Subjects

A total of 120 healthy male Sprague-Dawlay rats (4-month-old) with a weight range of 270 g to 330 g at clean grade were chosen. All rats were purchased from Slac laboratory animal company (Shanghai, China) and raised at the room temperature between 21°C and 23°C. The clean grade animal facility had a constant humidity of 60% ± 5% and good ventilation. The rats were raised in a quiet environment with a normal circadian rhythm, unregulated eating and drinking schedules. At 1 week before the experiment, rats were raised in adaptive conditions.

Animal Grouping and Model Establishment

These 120 rats were randomly assigned to five groups (24 rats in each group): the control group, the sevoflurane (Hengrui Pharmaceutical, Shanghai, China) group, the sevoflurane + splenectomy group (injections of saline solution 0.5 h before the operation and 6 h after the operation), the PDTC group (PDTC injection of 50 mg/kg at the same time points as in the sevoflurane + splenectomy + PDTC group), and the sevoflurane + splenectomy + PDTC group (intraperitoneal injections of PDTC at 50 mg/kg, and injected at 0.5 h before the operation and 6 h after the operation). The treatment regime of each group is shown in Table I. PDTC (a specific inhibitor of NF-κB) was purchased from Sigma (St. Louis, MO, USA).

The establishment of the animal anesthesia model was showed as follows: anesthesia induction for 5 min by 80% oxygen and 3% sevoflurane and the mouth of rats were opened to expose the trachea. A 14 G venous indwelling needle (Braun Company, Melsungen, Hessen, Germany) was inserted into the trachea to complete the tracheal intubation. The indwelling needle was connected to the gas output port of the anesthesia machine (Kontron Company, Augsburg, Bayern, Germany), and was connected to a multifunction anesthesia detector (General Electric Medical system, Helsinki, Finland) through a three-way valve. The anesthetic vapor contained 80% oxygen and 2% sevoflurane, and was maintained at this concentration during the 2 h inhalation period. The rats

Table I. The establishment of the animal anesthesia/operation model.

Group	Treatment
Control	No anesthesia or splenectomy
Sevoflurane	Anesthesia by 2% sevoflurane
Sevoflurane + splenectomy	Anesthesia by 2% sevoflurane, spleen removal, intraperitoneal injection of saline
PDTC	No anesthesia or splenectomy, intraperitoneal injection of PDTC (50 mg/kg)
Sevoflurane + splenectomy + PDTC	Anesthesia by 2% sevoflurane, spleen removal, intraperitoneal injection of PDTC (50 mg/kg)

Note: PDTC = pyrrolidine dithiocarbamate.

maintained spontaneous breathing till the end of anesthesia and were put under observation for 10 min. After full recovery from the anesthesia, the rats were raised in single cages.

The establishment of the operation model (splenectomy): it was similar to the anesthesia model till the completion of the tracheal intubation. Then the rats underwent routine skin disinfection procedures, and the layers of skin, muscle and peritoneal were cut through. After the ligation of the splenic artery including their branches and accompanying veins, splenectomy was performed. By the end, 5 ml of saline solution was injected into the abdominal cavity to compensate for the body fluid lost during the operation. The operation process was about 50 min, and the anesthesia was maintained for 2 h. After the operation, rats received the intramuscular injection of 3 million units penicillin (Sigma-Aldrich, St. Louis, MO, USA) to prevent infection. The rats were then placed in a quiet environment in single cages. The control group received no anesthesia or surgical treatments. During the anesthesia and operation, the vital signs of rats and inhaled gas were monitored.

Electrocardiogram (ECoG)

One day before the anesthesia or operation, the rats were deeply anesthetized by inhaling 3% sevoflurane (Hengrui Pharmaceutical, Shanghai, China) for 1 min (short-time deep anesthesia by sevoflurane does not affect hippocampal functions and biochemical changes). During the operation, the head of rats was immobilized on the framework of a Jiangwan C stereotactic instrument (Model I, Chengdu Instrument Factory, Sichuan, China). The skin was disinfected using conventional methods. A sagittal incision was cut along the midline of rat head, and copper screws (Chengdu Instrument Factory, Sichuan, China) were buried through the skull and into the frontal and parietal cortex. The ECoG signals were recorded by connecting the cortical electrodes with the ECoG instrument and using the ear circumference as a reference electrode. The ECoG signals of each group were recorded and saved by an RM6240 multi-channel physiological signal acquisition and processing system (Chengdu instrument factory, Sichuan, China). The system parameters were: gain 8000 ×, sampling frequency 400 Hz, low-frequency filter 40 Hz, time constant 0.2 s. The changes of ECoG signals in each group before and after anesthesia and operation, as well as 0 h and 6 h after drug treatments were used during the experiments.

Behavioral Observation

The behavioral observation started from the 7th day after the operation, using the DMS-2 Morris water maze testing system (RWD life science, Guangdong, Shenzhen, China) and trained the spatial memory in rats for 5 consecutive days. The system consisted of a rat behavior test system, an image acquisition system and a computer data processing and analysis system. The Morris water maze was 0.5 m in height, and 1.2 m in diameter. A black cylindrical bucket with a constant temperature-heating device was installed at the bottom of the maze. There were no landmarks in the water maze, and the camera was placed in the maze. During the tests, the environment was kept quiet, and the temperature was maintained between 22°C and 25°C.

The procedures during the place navigation test were: the water area was divided into 4 quadrants, and the rats were randomly put into one of the water quadrants facing the barrel wall. If a rat found the platform within 90 s and stayed on the platform for 5 s, the duration of its swimming was recorded as its escape latency; if a rat could not find the platform in the 90 s, the rat was then guided to the platform and stayed for 15 s, and the escape latency was set as 90 s. The experimental procedures were repeated for multiple times and the average value was calculated. The values were recorded for day 7, 9, 11, 13, 15 and 17 after the operation.

The procedures for space exploration experiments were: the time *ratio* (%) during swimming in the target quadrant was measured. On day 8, 10, 12, 14, 16 and 18 after the operation, the platform was removed and the rats were put into the target quadrant facing the barrel wall. In the following 60 s, the swimming path of the rat was observed, and the proportion of time (%) when the rat was still in the original quadrant was calculated.

Tissue Collection

Rats in each group were divided into two subgroups, with each subgroup contained 12 rats. At 24 h after the operation, 6 rats were used for hippocampal tissue isolation and biochemical measurements, and the hippocampus tissues in the other 6 rats were fixed for tissue sections. In the other subgroup, the 12 rats underwent animal behavior tests, and were sacrificed 2 days after the tests (i.e., 20 days after the operation). During the dissection, the rats inhaled 3% sevoflurane for 1 min to achieve deep anesthesia (i.e., short-

time sevoflurane anesthesia does not affect hippocampal and biochemical functions). The limbs of rats were then immobilized, their chest cavities quickly opened to expose the hearts. A 16 G venous puncture needle (Braun company, Melsungen, Hessen, Germany) were placed into the aorta by puncturing through the left ventricle, the right auricula dextra was cut and quickly infused with 200 ml of cold saline. The rats were then rapidly decapitated with their brains removed. The brain tissues were quickly transferred onto ice made from double distilled water. The hippocampal tissues were quickly isolated, rinsed with 4°C sterile water, dried and placed in liquid nitrogen before frozen in a -80°C freezer. For tissue slides, the hippocampal tissues were anesthetized and rapidly perfused with heparin saline (Boster, Hubei, Wuhan, China), and then fixed with 200 ml of 4% poly formaldehyde (Boster, Hubei, Wuhan, China).

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

The total RNA in the hippocampal tissues of the rat was extracted by Trizol (Invitrogen, Carlsbad, CA, USA) according to the instructions. The RNA purity and concentration were measured by a NanoDrop2000 spectrophotometer (Thermo, Waltham, MA, USA). The PCR primers were designed using the Primer 5.0 design software according to the gene sequence published in the Genbank database and were then synthesized (Sangon, Shanghai, China). The PCR reaction primers are listed as follows (Table II). The PCR was performed using an ABI prism 7500 real-time PCR system (ABI, Foster City, CA, USA), with the reaction mixture prepared according to the instructions of the SYBR Premix EX TaqII Kit (Takara, Dalian, Liaoning, China). The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. Each gene in the samples was measured in triplicates. The reliability of the PCR results was verified using the dissolution curve. The cycle threshold (Ct, which is the inflection point on the amplification power curve), ΔCt = Ct (target gene) – Ct (internal control), ΔΔCt = ΔCt (experimental group) – ΔCt (control group) were calculated, and the gene expression values were calculated as $2^{-\Delta\Delta Ct}$ [15].

Western Blotting

The total proteins in the cryopreserved hippocampal tissues were extracted using Trizol (Invitrogen, Carlsbad, CA, USA) according to the extraction steps written in the manual. The protein concentrations were measured by a BCA Kit (Beyotime Biotechnology, Shanghai, China). The extracted proteins were denatured by adding a buffer solution and boiled for 10 min at 100°C. The proteins were then separated using 10% polyacrylamide gel electrophoresis (PAGE) (Boster, Hubei, Wuhan, China). During PAGE, each well was added with 30 μg of samples, and the electrophoresis voltages were 60 V in concentrated gels for 45 min, and then changed to separation gels for 1 h at 120 V. The membrane used was polyvinylidene fluoride (PVDF). The wet transfer was used with a transferring voltage of 250 mA. The operation time of transfer was 2 h, and sealed at room temperature and in 5% bovine serum albumin (BSA) for 1 h. The primary antibodies were all used at a 1:1000 dilution: NF-κB-P65, Histone 3, p-IκBα, t-IκBα, IL-1β, IL-6, TNF-α and GAPDH (1: 1000; Abcam, UK). During antibody staining, the samples were incubated overnight at 4°C. After the staining, the samples were rinsed 3 times with a tris-buffered saline and tween 20 (TBST) solutions, with each rinse taking 5 min. The samples were

Table II. The primers for real-time PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
NF-κB-p65	CAGGACCAGGAACAGTTCGAA	CCAGGTTCTGGAAGCTATGGAT
IκBα	CGTGTCTGCACCTAGCCTCTATC	GCGAAACCAGGTCAGGATTC
IL-1β	CTCCATGAGCTTTGTACAAGG	TGCTGATGTACCAGTTGGGG
IL-6	TCTCTCCGCAAGAGACTTCC	TTCTGACAGTGCATCATCGC
TNF-α	TGACCCCCATTACTCTGACC	GGCCACTACTTCAGCGTCTC
GAPDH	TGCCCCCATGTTTGTGATG	GTGGTCATGAGCCCTTCCA

Notes: PCR = polymerase chain reaction; NF-κB = nuclear factor kappa B; IL = interleukin, TNF-α = tumor necrosis factor-alpha; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

then developed using a chemical luminescence reagent ECL (Beyotime Biotechnology, Shanghai, China), and the ImageJ software analyzed the grey values of the target bands.

Immunohistochemistry and Immunofluorescence Assay

The hippocampal tissues fixed by 4% poly formaldehyde were dehydrated by gradient alcohols, embedded in paraffin, and prepared into tissue slides with a thickness of 5 μm. The procedures for the immunohistochemistry treatments were: the tissue sections were dewaxed using general routines, and then immersed in 3% H_2O_2. After washed with phosphate-buffered saline (PBS), the samples were sealed in a sealing solution for 1 h. A volume of 150 μl solution containing anti-IgG primary antibodies (Vector, Burlingame, CA, USA; 1:100 dilution) was added to each slide, and the slides were incubated at 4°C overnight. The slides then were washed with PBS and added to 150 μl of biotinylated secondary antibodies (Vector, Burlingame, CA, USA; 1:500 dilution), and incubated at room temperature for 2 h. Finally, the slides were incubated at room temperature for 2 h with 150 μl of SABC solution in an immunohistochemical kit (Boster, Hubei, Wuhan, China), and developed in 150 μl of DAB. The slides were then sealed and photographed under an Olympus CX23 microscope. The blood-brain barrier (BBB) permeability in rat hippocampus was evaluated.

The procedures for the immunofluorescence assays were: the tissue sections were immersed in 3% H_2O_2. After sealing, 150 μl of anti-Iba-1 primary antibody (1:100 dilution; Wako Chemical, Richmond, VA, USA) were dropped on each slide. The slides were then incubated overnight at 4°C. Subsequently, 150 μl of secondary antibody fluorescently labeled by NL557 (1:500 dilution, R&D Systems, Minneapolis, MN, USA) were added to each slide, and the slides were incubated at room temperature and in the dark for 1 h. At last, Hoechst33342 (1:1000 dilution; Thermo Scientific, Waltham, MA, USA) was added to the slides, and the slides were incubated in the dark for 5 min. The slides were then sealed in the dark using a fluorescence quencher named Vectashield mounting medium (Vector Labs, Burlingame, CA, USA) and observed within 4 h. The expression of microglia marker Iba-1 in the rat hippocampal region was observed under a fluorescence microscope, using ImageJ 1.47n software to quantify the relative expression of Iba-1 under double-blinded conditions. The results were presented using the area percentage occupied by the positive region.

Statistical Analysis

All data were analyzed using the SPSS 18.0 statistical software (SPSS, Chicago, IL, USA). The measurement data were presented using the mean ± standard deviation (SD) (± s). The comparisons among multiple groups were conducted by the one-factor analysis of variance (ANOVA), and the comparisons between two groups abiding the normal distribution were done by the *t*-tests. The counted data were presented in percentages and ratios, and were verified using the X^2-tests. A probability value of $p < 0.05$ indicated the difference was statistically significant.

Results

General Conditions

After the anesthesia/operation, the rats were raised in single cages and their conditions were monitored, including their physical activities, eating and breathing status, piloerection, diarrhea, enophthalmos, and urine volumes. The observations showed that the body weights of the rats in the control group (305.0 ± 9.7 g), the sevoflurane group (301.5 ± 8.6 g), the sevoflurane + splenectomy group (302.8 ± 8.8 g), the PDTC group (299.7 ± 9.5 g) and the sevoflurane + splenectomy + PDTC group (304.1 ± 10.1 g) had no significant difference. During the anesthesia/operation, the vital signs of the rats were stable. There were no cases of hypoxemia or hypotension, and all rats survived throughout the entire experiment.

ECoG Observation

The brain waves were divided into α (8-12 Hz), β (13-25 Hz), δ (0.5-4 Hz), and θ (4-8 Hz) waves. The rats in the control group were mainly presented with fast (α and β) waves. In the sevoflurane group, after the reflex in the rats disappeared, the percentages of slow waves (δ and θ) increased gradually. Before the treatment, the brain waves in all groups were mainly fast waves. At 0 h and 6 h after the treatment, the brain waves in the control group were still mainly α and β waves, but the brain waves in the sevoflurane group and the sevoflurane + splenectomy group were slower δ waves, and a suppression change occurred in a burst fashion. When

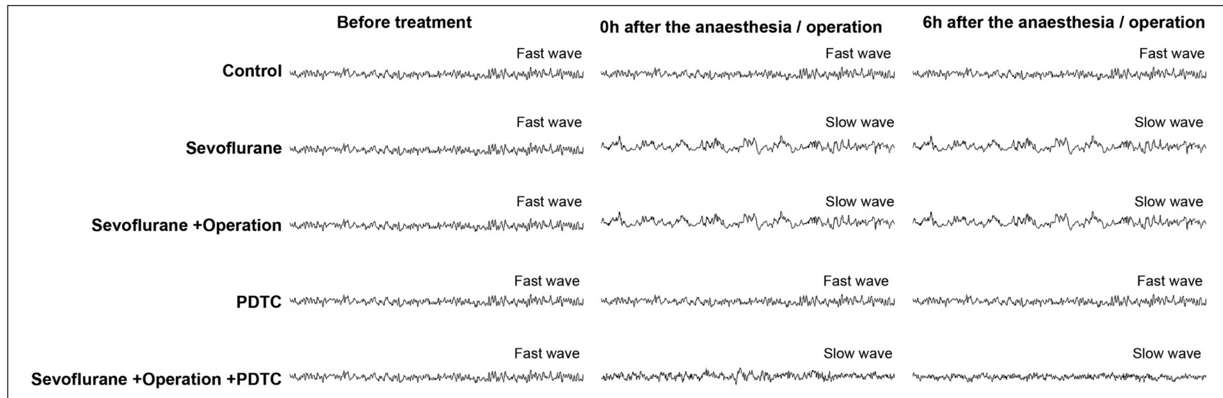

Figure 1. ECoG images of rats before and at 0 h and 6 h after the anesthesia/operation. ECoG, Electrocardiogram; PDTC, pyrrolidine dithiocarbamate.

compared with the control group, no significant effect on the brain waves in the PDTC group was observed. Compared with the sevoflurane + splenectomy group, the brain waves in the sevoflurane + splenectomy + PDTC group significantly changes in ECoG arousal pattern (Figure 1 and Table III).

Effects of Anesthesia/Operation Trauma on the Expressions of NF-κB, P65 and IκBα

At 24 h after the anesthesia/operation, the hippocampal tissues of rats in each group were obtained after detected by qRT-PCR. It was found that as compared to the control group, no significant change in the mRNA expressions of N*F-κB/P65* and *IκBα* was found in the sevoflurane and PDTC groups (both $p > 0.05$), while the mRNA expressions of N*F-κB/P65* and *IκBα* in the sevoflurane + splenectomy group were significantly increased (both $p < 0.05$). Also, the mRNA expressions of N*F-κB/P65* and *IκBα* in the sevoflurane + splenectomy + PDTC group decreased significantly in comparison to the sevoflurane + splenectomy group (both $p < 0.05$) (Figure 2A, 2B). The protein expressions of NF-κB/P65 and IκBα were measured with Western blotting, and the results were similar to those from the qRT-PCR measurements (Figure 2C, 2D). These results indicated that the surgical trauma significantly up-regulated the expressions of NF-κB/P65 and IκBα in the hippocampus, while PDTC treatment significantly alleviated the effects caused by surgical trauma.

Effects of Anesthesia/Operation Trauma on the Expressions of Inflammatory Cytokines

At 24 h after the anesthesia/operation, the hippocampal tissues of rats in each group were obtained. The measurements by the qRT-PCR showed that as compared with the control group, the mRNA expressions of *IL-1β*, *IL-6* and *TNF-α* in the sevoflurane and the PDTC groups showed no significant change (all $p > 0.05$), while the mRNA expressions of *IL-1β*, *IL-6* and *TNF-α* in the sevoflurane + splenectomy group significantly increased (all $p < 0.05$). As compared to the

Table III. The percentage (%) of δ waves in the ECoG graphs before and at 0 h and 6 h after anesthesia/operation.

Group	Before anesthesia/ operation	0 h after anesthesia/ operation	6 h after anesthesia/ operation
Control	21.18 ± 9.41	22.54 ± 9.52	22.89 ± 9.57
Sevoflurane	22.09 ± 10.12	68.35 ± 14.11*	57.05 ± 13.91*
Sevoflurane + splenectomy	21.34 ± 9.43	69.03 ± 14.2*	58.19 ± 14.06*
PDTC	22.39 ± 9.76	23.11 ± 9.47	23.86 ± 9.58
Sevoflurane + splenectomy + PDTC	21.27 ± 9.97	32.40 ± 13.28#	29.72 ± 13.05#

Notes: ECoG = Electrocorticogram; PDTC = pyrrolidine dithiocarbamate; *, $p < 0.05$ as compared to the control group; #, $p < 0.05$ as compared to the sevoflurane + splenectomy group.

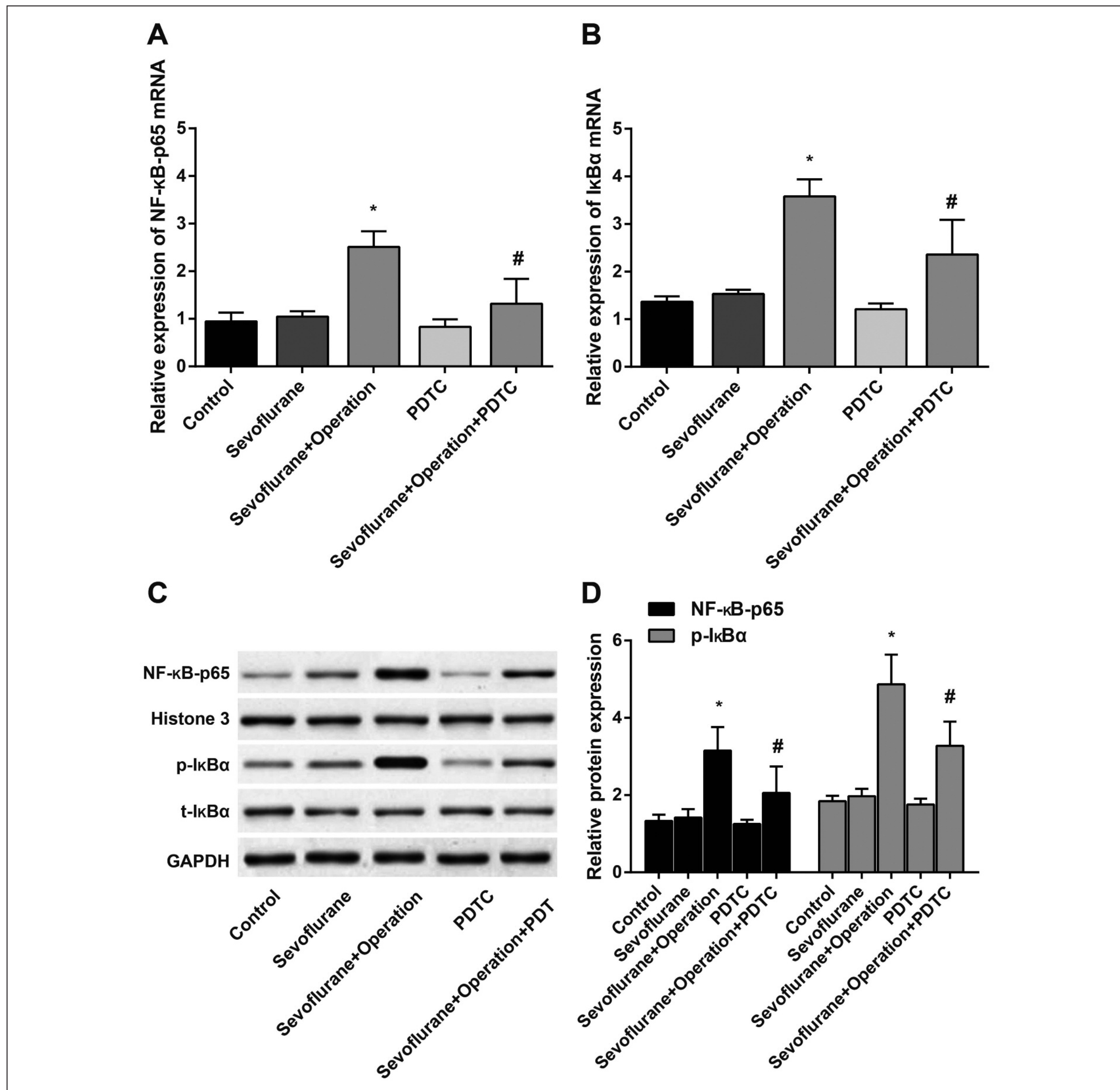

Figure 2. The expressions of NF-κB, P65 and IκBα in hippocampal tissues of rats after the anesthesia/operation. ***A,*** qRT-PCR showed the mRNA expression of NF-κB/P65; ***B,*** qRT-PCR showed the mRNA expression of IκBα; ***C,*** Western blotting showed the protein expression of NF-κB/P65; ***D,*** Western blotting showed the protein expression of IκBα. *, $p < 0.05$ compared with the control group; #, $p < 0.05$ compared with the sevoflurane + splenectomy group; PCR, polymerase chain reaction; NF-κB, nuclear factor kappa B.

sevoflurane + splenectomy group, the mRNA expressions of *IL-1β*, *IL-6* and *TNF-α* in the sevoflurane + splenectomy + PDTC group decreased (all $p < 0.05$) (Figure 3A, 3B). The measurements by the Western blotting showed similar results for the protein expressions of IL-1β, IL-6 and TNF-α in the nucleus (Figure 3C, 2D). These results indicated that the surgical trauma significantly up-regulated the expressions of inflammatory cytokines in the hippocampus, while PDTC treatment significantly alleviated the effects caused by surgical trauma.

Effect of Anesthesia/Operation Trauma on the Expressions of Microglia marker Iba-1 in Hippocampus

At 24 h after the anesthesia/operation, the hippocampal tissues of rats in each group were

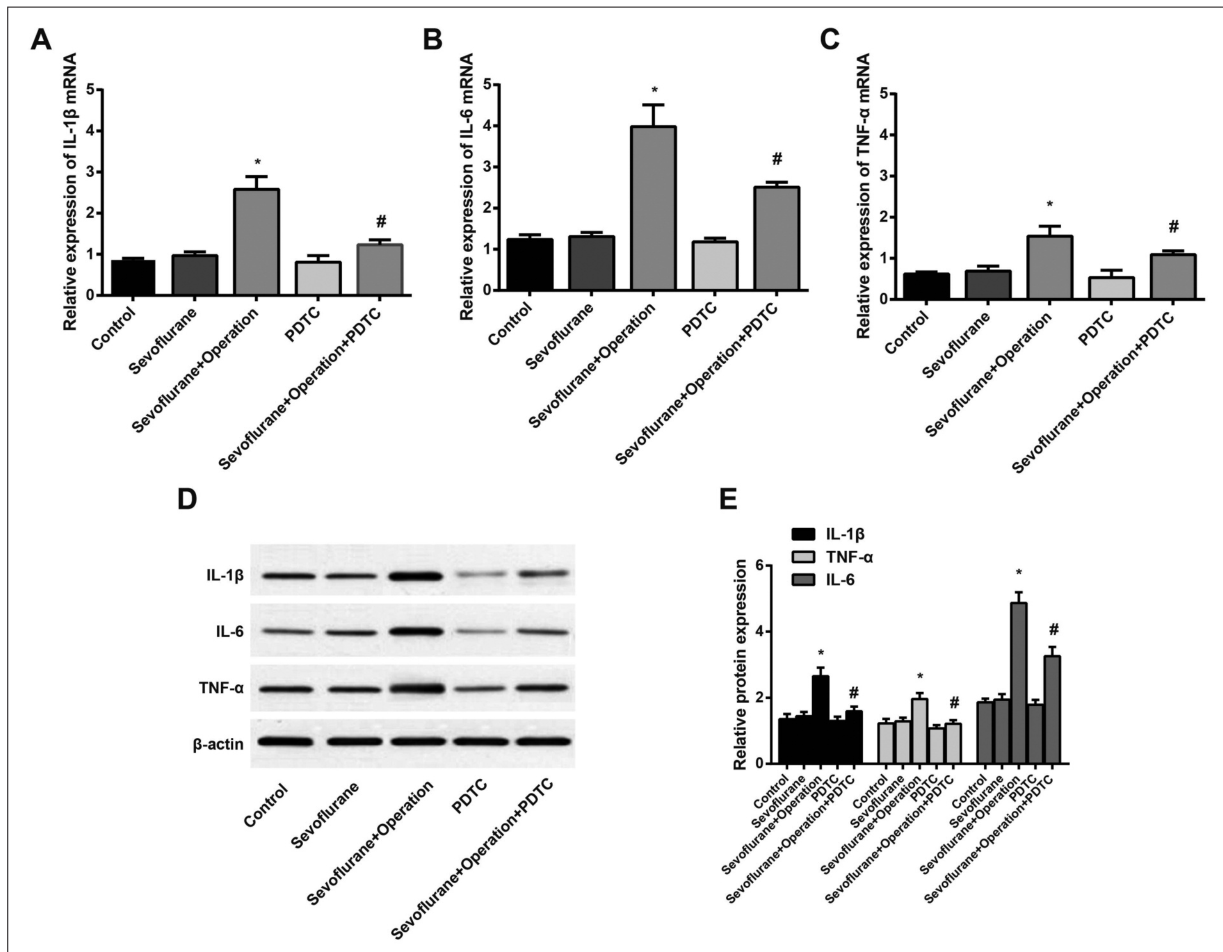

Figure 3. The mRNA and protein expressions of IL-1β, IL-6 and TNF-α in hippocampal tissues of rats after the anesthesia/operation. ***A,*** The mRNA expression of IL-1β detected by qRT-PCR; ***B,*** The mRNA expression of IL-6 detected by qRT-PCR. ***C*** The mRNA expression of TNF-α detected by qRT-PCR; ***D,*** The protein expressions of IL-1β, IL-6 and TNF-α detected by Western blotting; ***E,*** The grey value analysis of IL-1β, IL-6 and TNF-α protein expressions. *, $p < 0.05$ compared with the control group; #, $p < 0.05$ compared with sevoflurane + splenectomy group; IL, interleukin; PCR, polymerase chain reaction; TNF-α, tumor necrosis factor-alpha.

obtained. The expressions of microglial cell marker Iba-1 in the dentate gyrus (DG) and CA3 regions of rat hippocampus were measured by immunofluorescence assays. The results showed that as compared with the control group, the expression of Iba-1 showed no significant change in the sevoflurane and PDTC group ($p > 0.05$), while the Iba-1 expression in the sevoflurane + splenectomy group significantly increased ($p < 0.05$). As compared to the sevoflurane + splenectomy group, the Iba-1 expression in the sevoflurane + splenectomy + PDTC group significantly decreased ($p < 0.05$). The change of Iba-1 expression in the hippocampal DG region was similar to that in CA3 region (Figure 4).

Effect of Anesthesia/Operation Trauma on the Permeability of BBB in Rat Hippocampus

At 24 h after the anesthesia/operation, the hippocampal tissues of rats in each group were obtained. The BBB permeability in the hippocampal CA3 area was evaluated by IgG immunohistochemical assays. It was found that as compared with the control group, the treatments by sevoflurane or PDTC alone did not affect the BBB permeability (both $p > 0.05$), and the BBB permeability in the sevoflurane + splenectomy group significantly increased ($p < 0.05$). As compared to the sevoflurane + splenectomy group, the BBB permeability in the sevoflurane + splenectomy + PDTC group significantly reduced ($p < 0.05$) (Figure 5).

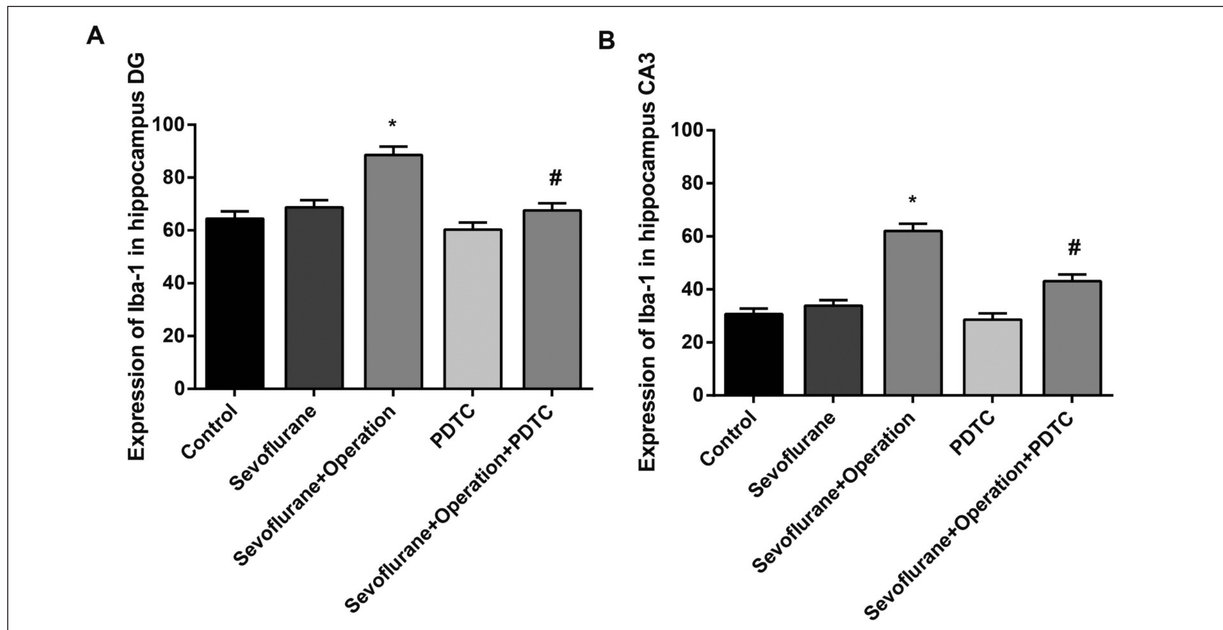

Figure 4. The expression of microglia marker Iba-1 in the DG and CA3 regions of rat hippocampus after the anesthesia/operation. ***A,*** The Iba-1 expression in rat hippocampal DG area; ***B,*** The Iba-1 expression in the CA3 region of rat hippocampus; *, $p < 0.05$ as compared to the control; $^{\#}$, $p < 0.05$ as compared with the sevoflurane + splenectomy group; Iba-1, ionized calcium-binding adapter molecule 1; DG, dentate gyrus.

These results indicated that the surgical trauma significantly increased the BBB permeability in the hippocampus, while PDTC treatment significantly inhibited the effects caused by surgical trauma.

Behavioral Tests

At different time points, the treatments by sevoflurane anesthesia or PDTC alone showed no significant effect on rat escape latency as compared with the control group (both $p > 0.05$), and the escape latency in the sevoflurane + splenectomy group significantly increased ($p < 0.05$). As compared to the sevoflurane + splenectomy group, the escape latency in the sevoflurane + splenectomy + PDTC group decreased significantly ($p < 0.05$). With the extension of training time, the escape latency in all groups decreased significantly except for the sevoflurane + splenectomy group (all $p < 0.05$) (Figure 6). The results from the space exploration experiments showed that as compared to the control group, the treatments by sevoflurane anesthesia or PDTC alone showed no effect on rat residence time in the target quadrant (both $p > 0.05$), and the residence time in the sevoflurane + splenectomy group decreased significantly ($p < 0.05$). As compared to the sevoflurane + splenectomy group, the residence time in the sevoflurane +

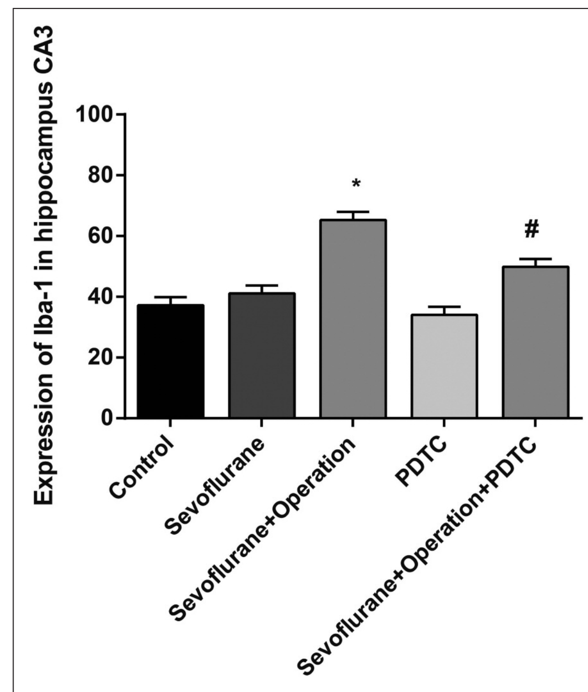

Figure 5. The BBB permeability in the CA3 zone of rat hippocampus among different groups after the anesthesia/operation. *, $p < 0.05$ as compared to the control group; $^{\#}$, $p < 0.05$ as compared to the sevoflurane + splenectomy group; PDTC, pyrrolidine dithiocarbamate; BBB, blood brain barrier.

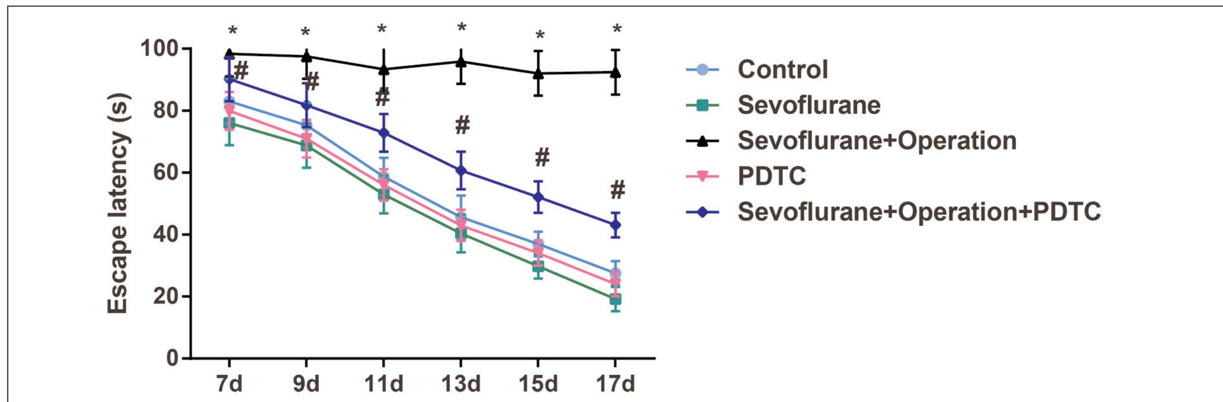

Figure 6. The differences in rat escape latency at different time points after the anesthesia/operation. PDTC, pyrrolidine dithiocarbamate; *, compared with control group, $p < 0.05$; #, compared with sevoflurane + splenectomy group, $p < 0.05$.

splenectomy + PDTC group increased significantly ($p < 0.05$). With the extension of training time, the rat residence time in the target quadrant increased in all groups except for the sevoflurane + splenectomy group ($p < 0.05$) (Table IV). These results indicated that the surgical trauma significantly damaged the behavioral functions of rats, while PDTC treatment could eliminate the effects caused by surgical trauma.

Long-term Effect of Anesthesia/Operation Trauma on the Expression of Iba-1

At day 20 after the behavior tests, the hippocampus tissues from the rats were collected and evaluated by the immunofluorescence assays. The results showed that the expression of microglial markers Iba-1 in rat hippocampus DG and CA3 regions were similar among the sevoflurane group, the sevoflurane + splenectomy group, and the control group (all $p > 0.05$) (Figure 7). These results suggested that in the long-term, the anesthesia/operation trauma had no significant effect on the expression of Iba-1 in hippocampal DG and CA3 areas.

Long-term Effect of Anesthesia/Operation Trauma on the Expressions of Inflammatory Cytokines

At day 20 after the behavior tests, the hippocampus tissues from the rats were collected and evaluated by qRT-PCR and Western blotting. The experimental results showed that the expressions of IL-1β, IL-6 and TNF-α in rat hippocampus were not different among the sevoflurane group, the sevoflurane + splenectomy group and the control group (all $p > 0.05$) (Figure 8). These results suggested that in the long-term, the anesthesia/operation trauma had no significant effect on the expression of inflammatory cytokines in rat hippocampus.

Discussion

In this study, the relationship between NF-κB/P65 signaling pathway and the regulation of cognitive impairment was studied. Experimental results showed that after sevoflurane anesthesia and surgical treatment, a majority of rat brain

Table IV. The results of space exploration experiments (% of the time stayed in the target quadrant).

Group	Day 8	Day 10	Day 12	Day 14	Day 16	Day 18
Control	21.3 ± 4.2	28.9 ± 4.0	38.6 ± 4.3	45.3 ± 5.1	53.0 ± 5.4	60.1 ± 5.5
Sevoflurane	20.9 ± 4.5	28.2 ± 4.1	37.9 ± 4.2	44.6 ± 5.4	52.9 ± 5.3	59.8 ± 5.1
Sevoflurane + splenectomy	13.0 ± 3.1*	13.5 ± 3.2*	13.9 ± 3.8*	14.3 ± 3.7*	14.7 ± 3.5*	14.9 ± 3.8*
PDTC	21.7 ± 4.9	29.1 ± 4.2	38.1 ± 4.1	45.7 ± 5.3	53.6 ± 5.2	61.2 ± 5.4
Sevoflurane + splenectomy + PDTC	18.5 ± 5.3#	24.9 ± 5.1#	32.0 ± 4.9#	40.8 ± 5.4#	49.2 ± 5.3#	56.6 ± 5.2#

Notes: PDTC = pyrrolidine dithiocarbamate; *, $p < 0.05$ as compared to the control group; #, $p < 0.05$ as compared to the sevoflurane + splenectomy group.

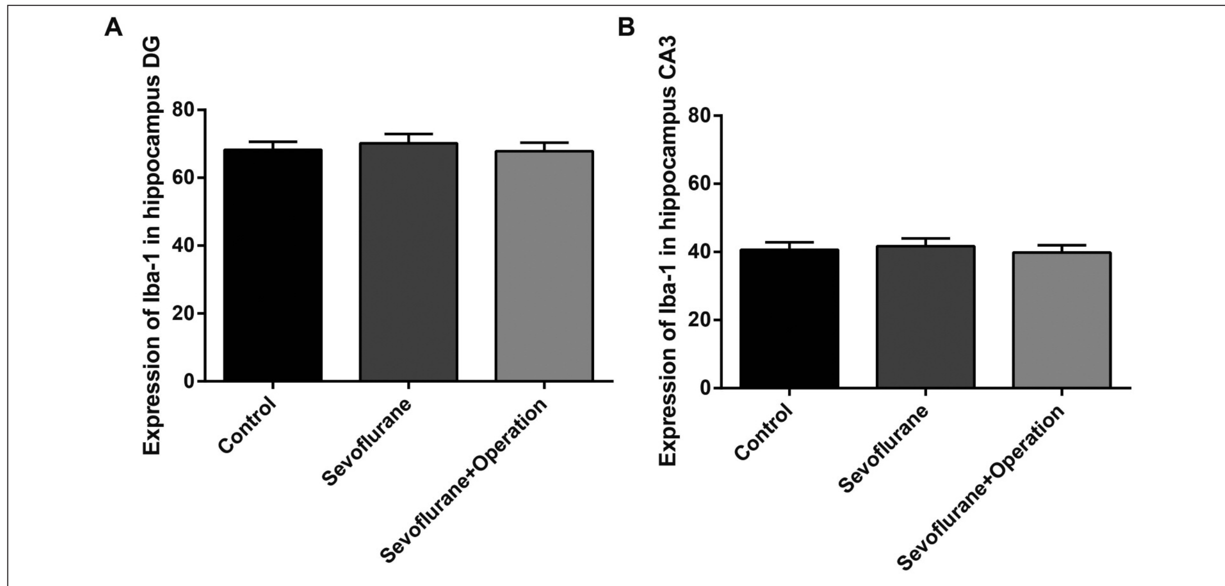

Figure 7. Long-term expression of Iba-1 in the rat hippocampal DG and CA3 regions after the anesthesia/operation. ***A,*** The expression of Iba-1 in the DG area; ***B,*** The expression of Iba-1 in the CA3 region; PDTC, pyrrolidine dithiocarbamate; DG, dentategyrus; Iba-1, ionized calcium-binding adapter molecule 1.

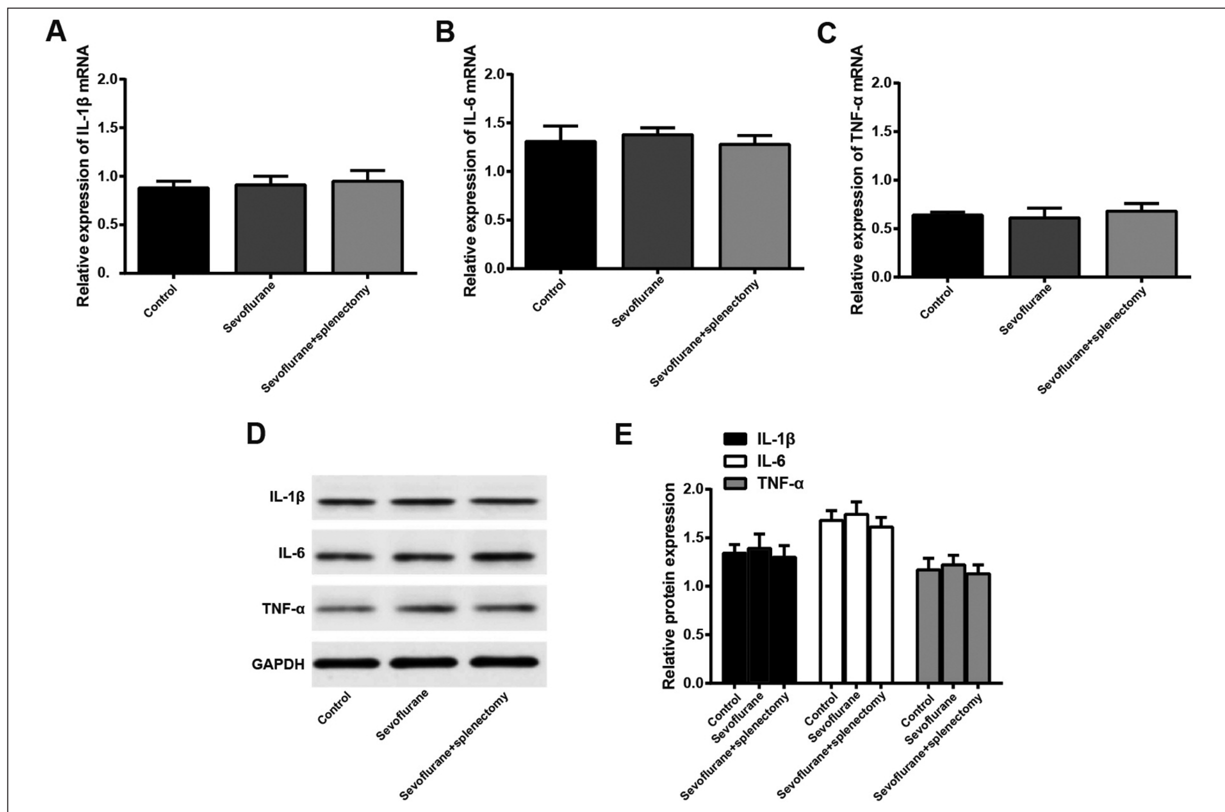

Figure 8. Long-term expressions of IL-1β, IL-6 and TNF-α in rat hippocampus after the anesthesia/operation. ***A,*** The expression of IL-1β detected by qRT-PCR; ***B,*** The expression of IL-6 detected by qRT-PCR. ***C,*** The expression of TNF-α detected by qRT- PCR; ***D*** The protein expressions of IL-1β, IL-6 and TNF-αdetected by Western blotting; ***E,*** The analysis of grey values based on the expressions of IL-1β, IL-6 and TNF-α protein in D; IL, interleukin; PCR, polymerase chain reaction; TNF-α, tumor necrosis factor-alpha; PDTC, pyrrolidine dithiocarbamate.

waves significantly changed into slow waves. However, this situation was alleviated after the rats treated with PDTC, which inhibits the NF-κB/P65 signaling pathway, and the majority of brain waves changed to fast waves. Thus, it is suggested that the activation of NF-κB/P65 signaling pathway induced by sevoflurane anesthesia/operation could decrease the brain activity and cause the symptoms of cognitive impairment.

According to previous research[16], the brain waves in the patients with cognitive impairment were significantly different from those in normal people. The experimental results from this study showed that such differences may be affected by the NF-κB/P65 signaling pathway. In the process of surgical treatments, the use of anesthetics such as sevoflurane on the one hand can increase the expression of IκB, while on the other hand the negative feedback mechanisms by IκB can inhibit the activity of NF-κB and slightly reduce its damages[17]. However, during the operation, the process of ischemia and reperfusion can cause the IκB to be phosphorylated and degraded, and hence activate the NF-κB factors[18]. Therefore, as compared to the normal rats, it is reasonable to observe that the treatments by sevoflurane anesthesia and operation increased the expressions of NF-κB in this study. As the results, the frequencies of rat brain waves decreased, which affected their escape latency and residence time on the platform. Also, treatment by PDTC inhibited the NF-κB signaling pathway, and recovered the brainwave activity, normalized the escape latency and the residence time on the platform.

This study also proved that the increase in the expression of NF-κB/P65 also increases the expression of inflammatory factor Iba-1, and hence increasing the permeability of BBB, which in turn affects the behavior of rats. It has been shown that in the mature nervous system, the activation of NF-κB was achieved through the synaptic signals, which played important roles in work, learning and memory processes[19]. The activation of NF-κB and the regulation of related gene expressions may be an important way to produce memory (20). Meanwhile, NF-κB is involved in the coding and functions of most proteins and genes related to neural plasticity[21]. Therefore, NF-κB plays an important role in the nervous system. Past research[22] has shown that the expression and activity of NF-κB/P65 increased with the increase of inflammatory response. These results were consistent with the results from this study, which found the expressions of IL-1β, IL-6 and TNF-α typically increased after the operation. In the present study, the levels of inflammatory factors were significantly decreased by using PDTC to inhibit NF-κB, suggesting that the expressions of these inflammatory cytokines were indeed correlated with NF-κB. Iba-1 is a calcium binding protein of 147 amino acids, which is usually considered a marker for microglia cells[23]. Iba1 is also reported to have utilized some microglial-specific antibodies to present the morphological characteristics of microglial cells[24]. The activated microglial cells released a variety of inflammatory factors, which affected tissue survival and repair[25]. Moreover, a previous study[26] supported that NF-κB is central in regulating the expressions of some genes and in synthesizing several proteins that participated in some immune response and inflammatory processes, and IκBα is an important regulator of the activity of NF-κB in glial cells. Therefore, it is reasonable to assume that the increase in the expression of NF-κB/P65 also increases the expression of inflammatory factor Iba-1.

The BBB can regulate the metabolic activities in the brain, and control the liquid flows from and into the brain[27]. Under the influence of some factors including angiogenesis factors, leukocyte adhesion factors and inflammatory factors, they could lead to the collapse of BBB functions, resulting in brain edema and aggravated inflammatory injury[27]. The postoperative recovery results from this research also suggested that in the long term, the operation and the anesthesia treatments by sevoflurane would not impact the expression of Iba-1 and the functions of BBB. Therefore, the increase in NF-κB expression caused by the operation was the main reason for certain damages to the nervous system and cognitive impairment. Inhibition of NF-κB activity by PDTC could help to recover the brain activity and cognitive functions.

Conclusions

These results suggest that the inhibition of NF-B/P65 signaling pathway may relieve POCD after sevoflurane anesthesia by down-regulating IκBα and inflammatory cytokines (IL-1β, IL-6 and TNF-α), Iba-1 expressions and the permeability of BBB. Thus, NF-κB/P65 signaling pathway could be a potential therapeutic target in

POCD after sevoflurane anesthesia. However, there were some limitations in this study. Firstly, the experiment results lacked the supports from relevant clinical data, so the conclusions drawn in this study may not be adequately applied in the general population. Also, the expressions of NF-κB/P65 were not measured again after the behavioral tests. Therefore, the correlation between NF-κB expression and temporal processes were not studied. Thus, these limitations shall be improved in future studies.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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