Remifentanil pretreatment attenuates brain nerve injury in response to cardiopulmonary bypass by blocking AKT/NRF2 signal pathway

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\section*{ABSTRACT}

\textbf{Objective:} This study aimed to explore the effect and mechanism of remifentanil on cardiopulmonary bypass (CPB)-induced cerebral nerve injury.

\textbf{Methods:} After pretreating with remifentanil, or dexmedetomidine (DEX), SD rats were subjected to the CPB for 2 h. The data of body temperature, blood gas and mean arterial pressure (MAP) and hematocrit (HCT) were recorded at different time points. The cerebral tissue water content of rats was determined and immunohistochemical (IHC) and H&E assays on the hippocampal CA1 region of rats was performed. The levels of interleukin (IL)-6, IL-10, soluble protein-100\textsuperscript{b} (S100\textsuperscript{b}) and neuron-specific enolase (NSE) were analyzed by ELISA, and those of the indexes for oxidative stress (malondialdehyde (MDA) and superoxide dismutase (SOD)) were detected by the commercial kits. Morris water maze was used to evaluate the learning and memory abilities. Western blot/qRT-PCR were used to detect the protein/mRNA expressions in hippocampus.

\textbf{Results:} CPB increased the levels/expressions of IL-6, IL-10, S100\textsuperscript{b}, NSE, MDA, cleaved caspase-3, Bax and decreased those of Bcl-2, SOD, p-AKT, HO-1, in serum and parietal cortex tissue, with increased brain water content, lesions in the hippocampal CA1 area, swimming distance, brain nerve injury and decreased escape latency, retention time on platform and times of crossing the platform of rats. The preconditioning of remifentanil or DEX partially attenuated CPB-induced injury and -decreased expressions on p-AKT and HO-1, while further promoting CPB-induced expression of nuclear Nrf2 expression and inhibiting that of cytoplasm Nrf2.

\textbf{Conclusion:} This paper demonstrates that remifentanil preconditioning could partially attenuate CPB-induced brain nerve injury of rats.

\section*{Introduction}

As the rapid development of cardiac surgery, cardiopulmonary bypass (CPB) and anesthesia technology, the number of cardiac surgery, especially CPB surgery, has significantly increased in China [1]. CPB could significantly increase the survival rate of patients suffered from cardiovascular surgeries [2–4]. However, during CPB, the release of inflammatory factors, microembolism, hypoperfusion and other factors can lead to hypoxic-ischemic changes in brain tissue, thereby resulting in brain tissue damage [5]. A observational study showed long-term persistent elevation of neurodegeneration markers in patients after elective cardiac surgery with the implementation of CPB [6]. Postoperative neurocognitive disorders have been reported as a leading cause of disability and death of patients following CPB [7]. Postoperative cognitive dysfunction (POCD) is a severe complication of CPB [8–10]. CPB-caused POCD was characterized by hippocampus apoptosis, which seriously limited the therapeutic efficacy and utilization of CPB in clinic [11]. The mortality rate of patients caused by postoperative postcentral nervous system (CNS) complications increases after CPB surgery [12]. In this sense, brain injury has seriously affected the ultimate effectiveness of open-heart bypass surgery. Therefore, investigations related to preventing brain damage in CPB have important clinical significance.

Remifentanil, a short-acting synthetic opioid analgesic, is an opioid widely used in general anesthesia [13]. It has attracted wide attention due to its good anti-oxidant stress property, and increasing discovery has provided the evidence that remifentanil has therapeutic effects in a variety of disease models caused by oxidative stress [14,15]. Besides, it has been shown to have cardiac-protective effects against ischemia-reperfusion injury [16–18]. The pretreatment with remifentanil can effectively improve the cerebral damage caused by cerebral ischemia reperfusion [19]. Moreover, high-dosage remifentanil preconditioning played a protective role on brain damage [20].

Nuclear factor E2-related factor 2 (Nrf2) regulates a range of antioxidant and cellular protective factors in response to oxidative stress, inflammation and neuronal apoptosis in...
brain injury [21–23]. Normally, Nrf2 is located in the cytoplasm and is transferred to the nucleus once activated [24]. Nrf2 shows strong antioxidant activity by binding to the antioxidant reaction element (ARE) in the promoter of the antioxidant genes and by enhancing the transcription of antioxidant stage-II enzymes such as HO-1 [25]. Akt, a serine/threonine protein kinase, is one of the most important and most widely studied protein kinases in the physiology and diseases of human beings, and the agent that alters the expressions of p-Akt relieves the neuronal injury via inhibiting the inflammation and apoptosis of neuronal cells [26]. Akt/Nrf2 pathway has been shown to be involved in the neuroprotective process in traumatic brain injury [27], and in cerebral ischemia injury in vitro and in vivo [28]. Besides, remifentanil has been reported to participate in Akt pathway [15]. Therefore, we speculated whether the Akt/Nrf2 pathway is also involved in the protective effects of Remifentanil against CPB-induced brain injury, and so conducted investigation on this hypothesis.

In this study, the role and effect of remifentanil in CPB-induced brain nerve injury were determined by examining the markers of oxidative stress and the apoptosis of neuronal cell, which further validated the effects of remifentanil on CPB-induced neurologic injury in vivo, providing a reliable theoretical basis for CPB-induced neurologic injury.

Materials and methods

Ethical statement

All animal experiments were conducted in accordance with the guidelines of the Chinese Council for Animal Care and Use, and the study was conducted at the First Affiliated Hospital of Guangxi Medical University. Our study has been ethically approved by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University (FAGMU201812357). All the researchers were dedicated to minimizing the discomfort of the animals throughout the experiment.

Animals and groups

Ninety-six healthy adult male SD rats (SPF, 8 months old, weighing 250–300 g) were selected as the experimental subjects in our study. Before the experiment, the rats were kept in a humidity room at 25 °C for several days and given adequate food and water. The animal house was maintained in a diurnal cycle (12 h/12 h) with the temperatures set at 25 °C and the humidity adjusted to 65%. The temperature of the room where the experiment was conducted was also set about 25 °C. The rats were randomly divided into six groups (16 rats/group): Sham operation group (Sham group), Model group (CPB group), Model + solvent group (CPB + DMSO group), Sham operation + Remifentanil preconditioning group (Sham + RPC group), Model + Remifentanil preconditioning group (CPB + RPC group), and model + Dexmedetomidine (DEX) group (CPB + DEX group). The rats in the sham group were used as the control group and were treated with intravenous catheterization and heparinization, whereas the CPB flow was not allowed. Rats in Sham + Remifentanil and in CPB + Remifentanil groups were injected with remifentanil for three times at an interval of 5 min (min) before CPB or sham surgery (injection rate: 1.2 µg min⁻¹ kg⁻¹. Capacity: 1 ml/5 min; Preconditioning time: 30 min; Remifentanil was dissolved with DMSO) (cat. No. 132539-07-2; Sigma-Aldrich, St. Louis, MO, USA) [29]. Rats in the CPB + DEX group were given an injection of 5 µg/kg DEX (cat. No. D1756, Sigma-Aldrich, St. Louis, MO, USA; purity: ≥98%, DEX was a neuroprotective drug) [30] and was dissolved with DMSO) 10 min before surgery, while those in the CPB + DMSO group were given an administration of DMSO (cat. No. ST038, Beyotime Biotechnology, Shanghai, China) 30 min before the surgery with the same dose and injection method as DEX. After 2 h, the CPB pipe was removed, and the experiment was finished. DMSO (4%) was used as a negative control and drug vehicle.

Animal anesthesia

Rats were anesthetized by intraperitoneal injection of 1% pentobarbital sodium (0.5 ml/100 g). The rats were fixed supine on the experimental operating table, and 16 G intravenous indwelling catheter was inserted into the trachea through the oral cavity for mechanical ventilation. Tidal volume was set at 3 ml/100 g, with the frequency at 60 times/min, and the ratio of suction and exhalation was adjusted to 1:1.5. The partial pressure of carbon dioxide (PaCO₂) was maintained in the range of 35–45 mm Hg throughout the experiment.

CPB model construction

The skin was cut lengthways after the shaving and disinfection in the anterior jugular area and the left and right inguinal areas. A 20G intravenous puncture needle was placed in the right external jugular vein to the right atrium, and the venous blood was drained by gravity to the blood reservoir. Femoral vein drainage was performed by placing the 22G intravenous puncture needle in the right femoral vein. 24 G intravenous puncture needles were placed in the left and right femoral arteries, respectively. The right femoral artery was perfused, and the left femoral artery was used for monitoring ambulatory blood pressure and blood gas. The veins (right femoral vein and the right external jugular vein to right femoral artery bypass) were used for CPB. The CPB circuit constitutes a venous reservoir, roller pump (Stockert SIII, Sorin, Germany), membrane oxygenator (MeicroPort, Dongguan, Guangdong, China), and aseptic duct with 4 mm in diameter. Before CPB, the circuit was primed with 13 ml of fresh heparinized blood from congenic rat, 5 ml of Ringer’s solution, and 5 ml of 4% hydroxyethyl starch solution. 480 s (s) after the Activated Clotting Time (ACT) of whole blood, rats with CPB were administered with 500 U/kg heparin. After CPB started, the perfusion flow was gradually increased, and the flow during CPB was stable at a rate of 100–120 ml/(kg·min). The mean arterial pressure in 75–100 mm Hg and Hematocrit (HCT) stability in 20–25% were used during CPB.
According to the results of blood gas analysis in discretion-ary use NaHCO₃ 5%, additional heparinized fresh blood, Ringer’s solution and hydroxethyl starch solution were added to supply the losses of fluid during experiment (such as loss of blood and urine during experiment, the blood specimen collection, evaporation and body liquid transferred to organization clearance and so on). The concentration of oxygen was set at 50~100%, and the oxygen flow was 300~400 ml/(kg·min). In the experiment, it was adjusted according to the results of blood gas to maintain the normal range. The anal temperature was maintained at 36.5 ± 1.0 °C in each rat using a self-made heating water blanket and a heating lamp. The experiment was finished after CPB had worked for 2 h. After CPB, the rats were housed in temperature-controlled cages (20 °C to 22 °C) until the spontaneous breathing was recovered and were freely accessed to 20% glucose drinking after 12 h surgery and freely accessed to food after 24 h surgery. The animals were given appropriate postoperative care consisted of antibiotics (1 day) and analgesic treatment (2 days). A schematic drawings of the CPB model construction was shown in Supplementary Figure 1A,B.

**Temperature recording and blood sample collection**

The body temperature, mean arterial pressure (MAP) and hematocrit (HCT) of rats at the indicated time points (pre-operative (T0), 1 h (T1) and 2 h (T2) after the construction of CPB model) was recorded and arterial blood (0.3 ml/time) was collected for the analysis on the blood gas, including partial pressure of oxygen (PaO₂) and arterial PaCO₂. The venous blood was collected for the determination on the levels of interleukin (IL)-6, IL-10, soluble protein-100β (S100β) and neuron-specific enolase (NSE) in the plasma. Venous blood samples were centrifuged at 1000 × g for 15 min, and the supernatant for the plasma was collected and stored at −20 °C. Finally, ELISA was used to determine the levels of each factor in the plasma.

**Morris water maze and space exploration experiment**

Two days after the surgery, Morris water maze (cat. No. DigBehv001, Shanghai Jiliang Software Technology Co. Ltd., Shanghai, China) was used to evaluate the postoperative learning ability and the memory retention of rats in each group. First, rats were acclimated to the water environment and trained for swimming for three days prior to the behavioral tests. The training for orientation navigation lasted for 6 days, to be specific, the rats were randomly placed into the water from four quadrants facing the wall of the pool for four times a day. Between the four trials, the rats were allowed to stay on the platform and rest for 15 s to provide time for the rats to observe the cues around them, and then, the next training was started. The swimming track of the rats was automatically recorded by the camera system and analyzed by the corresponding software. The maximum time limit for the rats to find the platform was set as 60 s. After the rats appeared on the stage, 5 s was regarded as the rats successfully found the hidden platform, and the recording of the camera system automatically stopped. All rats were ensured to have consistent training intervals. If the rats failed to find the hidden platform within 60 s, the camera system would automatically stop recording, and the escape latency (the time from entering the water to finding the hidden platform) would be recorded in 60 s. The rats were then guided to find the platform by the experimenter and were allowed to stay on the platform for 15 s. The experiment for the space exploration was carried out on the next day after orientation navigation was finished, where the platform was removed, and then the rats were put into the pool at any two entry points (except the quadrant of the platform), and their swimming track within 60 s was recorded. The average value of two attempts was recorded and taken as the experimental result of space exploration. The parameters, including the escape latency, times of crossing the platform, retention time on platform and swimming distance, were automatically recorded and analyzed using the system.

**Brain tissue collection**

Brain tissue samples (n = 8) were collected at the end of the CPB. The skull of rats was opened and the whole brain tissue was taken out. Then, the rats were placed on the surface of ice to separate the hippocampus on both sides. The right hippocampus was kept in the refrigerator with the temperature of −80 °C for the determination of western blot, while the left hippocampus was placed in the RNA preservation solution (cat. No. SR0020, Solarbio, Beijing, China) and then kept in the −80 °C until the determination of qRT-PCR. The levels of MDA and SOD in parietal cortex were determined by enzyme-linked immunosorbent assay (ELISA). The rest of the brain tissue, except for the hippocampus and cortex tissue, was used to determine the water content of the brain tissue.

**Immunohistochemical (IHC) and hematoxylin–eosin (H&E) staining assays**

Eight rats in each group were used, and then, the sternum of the rat was cut open to expose the heart and the aorta. The 20 G intravenous indwelling needle was placed at the apex of the heart in rats, and a small hole on the left ventricle was opened and the needle was inserted through the ventricle to extend about 5 mm straightly. Then, the abdominal aorta of the rats was blocked and the right atrium was cut open, and 100 ml of normal saline was administered into the tubule sleeve until the fluid flowing out of the right atrium was colorless, while a 4 °C 4% paraformaldehyde solution were injected into the tubule sleeve until the cervical rigidity became hard. After the rats were perfused with paraformaldehyde, the brains were removed and further fixed in 4% paraformaldehyde in PBS at 4 °C for 24 h, followed by being routinely dehydrated, cleared, paraffin-embedded, and sectioned at 5 μm thick. Subsequently, the fresh slices were placed in an oven at 65 °C for 3 h and affixed to glass slides for IHC staining assay, or H&E staining assay. For IHC staining
The needed slats were taken out of the aluminum foil bag after being balanced at room temperature for 48 h later. The tissue was taken to determine the dry weight of the brain tissue. The brain water content was quantified using the calculation formula: brain water content (%) = [(wet weight - dry weight)/wet weight] × 100%

**Determination of brain water content**

The brain tissue (n = 8) used to determine the brain water content was harvested firstly and the weight was immediately measured by an electronic balance, the results of which were deemed as the wet weight of the brain tissue. The brain tissue was then placed in a 55°C temperature chamber. 48 h later, the tissue was taken to determine the dry weight of the brain tissue. The brain water content was determined using an inverted microscope (Eclipse Ti-U, Nikon, Tokyo, Japan) under 100× and 200× magnification.

**Enzyme-linked immunosorbent (ELISA) assay**

The ELISA kits (IL-6 (cat. No. ab234570), IL-10 (cat. No. ab214566), S100β (cat. No. 69-80163) and NSE (cat. No. 69-30387)) used in this study were obtained from Abcam (Cambridge, UK) and MSKbio (Wuhan, China) according to the manufacturer’s instructions. Five random visual fields of the hippocampal CA1 area in each slice were photographed using an inverted microscope (Eclipse Ti-U, Nikon, Tokyo, Japan) under 100× and 200× magnification.

The brain tissue was then incubated at 37°C, followed by the filling of each well with washing liquid. After the quiescence for 1 min, the washing liquid on the slats were removed, and the slats were dried on the absorbent paper for 5 min in total. Substrate A and B were added to each well in a total volume of 50 µL, and the well was further incubated at 37°C for 15 min in the dark. The OD value of each well was determined at the wavelength of 450 nm by adding 50 µL stop solution of for 15 min.

**Oxidative stress detection assay**

The kits for the determination on the levels of MDA (cat. No. ab118970) and SOD (cat. No. ab65354) were purchased from Abcam. The steps for the determination on the levels of MDA were listed as follows: 3 mg of rat cerebral cortex tissue was added into 303 µL lysate (including 300 µL MDA lysate buffer and 3 µL butylated hydroxytoluene (BHT)), and the tissue was homogenized with a homogenizer, followed by being centrifuged in a centrifuge at 13000 × g for 10 min and the supernatant was collected. Afterwards, 600 µL thio-barbituric acid (TBA) solution were added into 200 µL standard and test samples and the MDA-TBA adduct was incubated at 95°C for 1 h and cooled to room temperature within an ice bath for 10 min. Then, 200 µL standard and test samples were transferred to the 96-well plates, and the OD value at 532 nm was detected and recorded by a microplate reader (iMark Microplate Reader, Bio-Rad, Hercules, CA, USA).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)**

Thirty milligram tissue samples were taken and ground into powder by liquid nitrogen. Total RNA in the tissues was extracted using the Trizol (cat. No. 15596-018, Invitrogen, Carlsbad, CA, USA) according to the protocols of the manufacturer. Then, cDNA was synthesized by the reverse transcription reaction kit (cat. No. RR037A, TaKaRa, Kyoto, Japan). According to the instructions provided by the kit, the equivalent amount of total RNA (approximately 500 ng) was taken and added with the 2 µL 5× gDNA Eraser Buffer, 1 µL gDNA Eraser and the supplement of RNase-free water, and the total volume was adjusted to 10 µL, and the product was placed for 5 min at room temperature. 2 µL 5× Prime Script Buffer, 6.5 µL RNase-free water, 1 µL RT Primer Mix, 0.5 µL Prime Script RT Enzyme Mix were added at the process of reverse transcription during PCR, the conditions of which were mentioned as follows: 37°C, 15 min, and 85°C, 5 s (s), and finally maintained at
4°C, while 10 μL SYBR Premix Ex Taq II (cat. No. RR820A, Takara, Kyoto, Japan), 0.8 μL for both forward primer and reverse primer (10 mM), 1 μL cDNA, and 6.4 μL ddH₂O were used for qPCR under the thermocycling conditions: 95°C, 30 s, followed by 40 cycles of 95°C, 3 s and 60°C, 30 s. The Ct value of the target gene and internal reference of the sample to be tested was determined as indicated above, and the relative expressions of genes were calculated by 2^(-ΔΔCt) method [31]. The primers were provided by Sangon (Shanghai, China) and listed in Table 1.

**Western blot**

The tissues were washed three times with normal saline and ground into powder with liquid nitrogen. One milliliter of RIPA lysis buffer (cat. No. P0013B, Beyotime, Shanghai, China) supplemented with 1% protease inhibitor (cat. No. P1030, Beyotime, China) was added to each sample. The samples were subsequently placed on ice for 40 min to fully decompose the protein into a 1.5-ml centrifuge tube. The protein was centrifuged at 12000 × g at 4°C for 5 min, the supernatant of which was separated and stored in the refrigerator at −80°C. The concentration of the extracted protein was determined by a BCA kit (cat. No. P0012, Beyotime, China). 30 μg protein and 4 μL marker (cat. No. PR1910, Solarbio, Beijing, China) were then separated on the SDS-PAGE (cat. No. P0012A, Beyotime, China). After the electric transfer of the protein samples, the PVDF membrane (cat. No. ISEQ00010/IPVH00010, MILLIPORE, Billerica, MA, USA) was rinsed with 1 × TBST for three times (5 min for each time), sealed using 5% skimmed milk powder and incubated in a shaking table at room temperature for 1 h. The corresponding primary antibodies (Cleaved caspase 3: cat. No. ab214430, 1:5000, Abcam; Bcl-2, cat. No. ab182858, 1:2000, Abcam; Bax, ab32503, 1:5000, Abcam; p-Akt, cat. No. ab214430, 1:5000, Abcam; Bcl-2, ab32503, 1:5000, Abcam; Akt, cat. No. ab8805, 1:500, Abcam; NRF2, cat. No. ab137550, 1:1000, Abcam; H3, cat. No. ab1791, 1:1000, Abcam; HO-1, cat. No. ab223349, 1:1000, Abcam; β-actin: cat. No. ab179467, 1:5000, Abcam) were diluted with TBST and were added to the membrane, for the incubation at 4°C overnight. The primary antibodies were discarded and the membrane was washed with TBST in a shaker for three times (5 min for each time). Select the appropriate infrared labeled secondary antibodies (Goat Anti-Rabbit IgG H&L (HRP): cat. No. ab6721, 1:10000, Abcam; Goat Anti-Mouse IgG H&L (HRP): cat. No. ab6789, 1:5000, Abcam) were selected for further incubation at room temperature for 2 h. TBST was used to wash the membrane for 3 times (5 min for each time), and the protein bands were visualized by the ECL solution (cat. No. WBKLS0500, EMD Millipore, Billerica, MA) and an image system (Bio-Rad, CA, USA). The gray value for each band was measured by ImageJ version 1.8.0 software (National Institutes of Health, Bethesda, MD, USA).

**Statistical analysis**

All data were expressed as the mean ± standard deviation. Statistical analysis was carried out using the SPSS 19.0 software (IBM, NY, USA). Statistical significance was determined by performing a one-way ANOVA followed by Tukey’s correction for multiplicity as appropriate. A value of p < .05 was considered to be statistically significant.

**Results**

**Remifentanil alleviated CPB-induced oxidative stress and brain injury**

First, we verified that CPB, remifentanil and DEX had no effect on experimental rats by measuring the body temperature and blood gas. According to Figure 1(A–D), there was no significant difference in body temperature, blood gas indexes ($P_{\text{O}_2}$ and $P_{\text{CO}_2}$), or MAP between the six groups. Statistical analysis was performed by an ANOVA followed by Tukey’s correction for multiplicity as appropriate. A value of $p < .05$ was considered to be statistically significant.

We further tested the brain water content and levels of the indexes for oxidative stress (MDA and SOD) in the parietal cortex tissue and observed the pathological conditions of the hippocampal CA1 tissue in the rats. The determination of brain water content suggested that the brain water content in rats of CPB group was higher than those in Sham group, while the pretreatment of remifentanil and DEX was able to reduce the CPB-induced increase on the brain water content (Figure 3(A); $p < .01$). Notably, in order to eliminate the interference of solvent, the group with the pretreatment of DMSO was established as well, and, no decrease was found on the levels of IL-6, IL-10, S100β and NSE in the serum of rats after the pretreatment of DMSO, compared with CPB group (Figure 2(A–D)). It could be shown and therefore summarized from the experimental results above that remifentanil and DEX could indeed alleviate CPB-induced brain injury.

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**Table 1.** Specific primer sequences for quantitative reverse transcription polymerase chain reaction.

| Gene     | Primer sequence                      | Species |
|----------|--------------------------------------|---------|
| Bcl-2    | 5′-GGTGAGGAGGACACTCTCTCA-3′          | rat     |
|          | 5′-GAGCGGCCCTTCAGAGACA-3′           |         |
|          | 5′-CCAGAACTGGCCGCGGT-3′             | rat     |
|          | 5′-TCAGCAGGAAGTCCAGTGT-3′           |         |
|          | 5′-ACTGCGATTGATGAGTCCT-3′           | rat     |
|          | 5′-CAGCAGTGTGTGGCAGATA-3′           |         |
Figure 1. CPB maintained intraoperative indicators. (A-E). The body temperature (A), PaO2 (B) and PaCO2 (C), MAP (D) and HCT (E) of the rats treated or untreated with DMSO, remifentanil and DEX was recorded at preoperative (T0), 1 h (T1) and 2 h (T2) after CPB model was constructed. CPB: cardiopulmonary bypass; DMSO: Dimethyl sulfoxide; RPC: remifentanil; DEX: dexmedetomidine; MAP: mean arterial pressure; HCT: hematocrit.

Figure 2. Remifentanil decreased CPB-induced elevation of IL-6, IL-10, S100β and NSE. (A-D). The levels of IL-6 (A), IL-10 (B), S100β (C) and NSE (D) in the serum of the rats treated or untreated with DMSO, remifentanil and DEX were measured by ELISA at preoperative (T0), 1 h (T1) and 2 h (T2) after CPB model was constructed. * vs. Sham, † vs. CPB + DMSO; ‡‡‡ or ‡‡‡‡ p < .001. CPB: cardiopulmonary bypass; DMSO: dimethyl sulfoxide; ELISA: enzyme-linked immune sorbent assay; RPC: remifentanil preconditioning; DEX: dexmedetomidine; IL: interleukin.
CPB + DMSO groups were scattered, irregular, and unevenly stained (Figure 3(B)). Also, the pretreatment of remifentanil or DEX results in more orderly arranged neurons in the CA1 area of the hippocampal hippocampus in rats, with a clear structure, and even staining, when compared with the CPB + DMSO group (Figure 3(B)).

**Remifentanil reduced latency in escape and improved memory in rats**

To examine the effects of remifentanil on the abilities of learning and memory of rats, rats in each group were subjected to the Morris water maze. According to the results, CPB could improve the time of escape latency and the swimming distance of rats and reduce the number of rats crossing the platform and the retention time on the platform, compared with Sham group (Figure 4(A–D); \( p < .001 \)). However, when compared with Sham group, the preconditioning using remifentanil in rats without CPB did not cause changes in the expressions of apoptosis-related factors, while in rats with CPB, the preconditioning of remifentanil reduced the expressions of Cleaved Caspase-3 and Bax and promoted that of Bcl-2 when compared to the CPB + DMSO group (Figure 5(A–C); \( p < .001 \)). The similar effects on the expressions of Cleaved Caspase-3, Bax, and Bcl-2 in rats with CPB were also evidenced after the preconditioning of remifentanil and DEX increased the numbers of crossing the platform and the retention time on the platform in rats with CPB (Figure 4(A–D); \( p < .001 \)).

**Remifentanil prevented CPB-induced apoptosis in the hippocampus and changes in the Akt/Nrf2 signaling pathway**

We subsequently set out to determine the effects of remifentanil on the expressions of apoptosis-related factors, and in accordance with the results, CPB increased the expressions of Cleaved caspase-3 and Bax in the hippocampal tissues, while inhibiting that of Bcl-2, compared with Sham group (Figure 5(A–C); \( p < .01 \), \( p < .001 \)). Meanwhile, when compared with Sham group, the preconditioning using remifentanil in rats without CPB did not cause changes in the expressions of apoptosis-related factors, while in rats with CPB, the preconditioning of remifentanil reduced the expressions of Cleaved Caspase-3 and Bax and promoted that of Bcl-2 when compared to the CPB + DMSO group (Figure 5(A–C); \( p < .001 \)). The similar effects on the expressions of Cleaved Caspase-3, Bax, and Bcl-2 in rats with CPB were also evidenced after the

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**Figure 3.** Remifentanil relieved CPB-induced neurologic injury. (A). The brain water content of the rats treated or untreated with DMSO, remifentanil and DEX was determined after the establishment of CPB model. (B). The neurons in the hippocampus of the rats treated or untreated with DMSO, remifentanil and DEX was identified by H&E after CPB model was constructed. scales: 200 μm and 100 μm; magnifications: \( \times 100 \) and \( \times 200 \). (C–D). The levels of MDA (C) and SOD (D) in the parietal cortex of rats treated or untreated with DMSO, remifentanil and DEX was measured by ELISA after the construction of CPB model. * vs. Sham, # vs. CPB + DMSO; ** or *** \( p < .01 \). CPB: cardiopulmonary bypass; DMSO: dimethyl sulfoxide; ELISA: enzyme-linked immune sorbent assay; H&E: hematoxylin–eosin staining; RPC: remifentanil; DEX: dexmedetomidine; MDA: malondialdehyde; SOD: superoxide dismutase.
preconditioning using DEX (Figure 5(A–C); \( p < .001 \)). We additionally explored the regulatory roles of remifentanil in the Akt/Nrf2 signaling pathway. As detailed in Figure 5(D–J), CPB could promote the expression of nuclear Nfr2 and reduce those of p-Akt, cytoplasm Nrf2, HO-1 as well as the ratio of p-Akt to Akt, but has no significant effect on the expression of Akt, compared with Sham group (\( p < 0.001 \)). The preconditioning of remifentanil and DEX were able to alleviate CPB-induced changes in the expressions of p-Akt, HO-1 and the ratio of p-Akt to Akt (Figure 5(D–F), 5(I–J); \( p < .001 \)). In addition, the preconditioning of remifentanil and DEX were able to further promote the expression of nuclear Nrf2 and reduced that of cytoplasm Nrf2 (Figure 5(G–H); \( p < .05 \), \( p < .001 \)). From IHC assay, the results showed that p-AKT and HO-1 were expressed in both the cytosol and nuclear compartments of neurons, but obviously expressed in sham, sham + RPC, CPB + RPC and CPB + DEX groups (Figure 6). Moreover, RPC or Dex reversed the inhibitory effect of CPB on p-AKT and HO-1 expressions (Figure 6).

**Discussion**

Remifentanil is a selective \( \mu \)-opioid receptor agonist that is also shown to have regulatory effect on neuronal activation, and it mainly circulates in the non-ionized region and rapidly crosses the lipid blood–brain barrier [16,32]. In this study, brain function was evaluated in a CPB rat model. The markers of brain injury, factors of oxidative stress inflammation and apoptosis, as well as Akt/Nrf2 signaling pathway-related proteins were examined to investigate the role of remifentanil in brain injury in CPB rats. This study reveals the potential mechanism of remifentanil pretreatment for CPB-induced brain injury.

In this study, the preconditioning with remifentanil alleviated CPB-induced cerebral nerve injury. In addition, the learning and memory abilities of rats with CPB were significantly reduced, whilst the remifentanil pretreatment had a good palliative effect. To further verify the results, we also examined the levels of serum brain function indicators S100\( \beta \) and NSE. During the damage of the cells within the central nervous system, S100\( \beta \) and NSE could leak out of cytological fluid into cerebrospinal fluid and then enter the blood through the damaged blood–brain barrier [30], suggesting that increased levels of S100\( \beta \) and NSE in the cerebrospinal fluid and blood are specific, and S100\( \beta \) and NSE can be utilized as the sensitive biochemical markers for the injury in CNS. In fact, according to the results proposed in our study, the levels of both S100\( \beta \) and NSE were increased in rats with...
Figure 5. Remifentanil reversed the inhibitory effects of CPB on the expressions of apoptosis- and Akt/NRF2 pathway-associated proteins. (A–C). The expressions of Cleaved caspase-3, Bax and Bcl-2 in the rats treated or untreated with DMSO, remifentanil and DEX were measured by western blot and qRT-PCR. β-actin was the internal reference of Cleaved caspase-3, Bax and Bcl-2. (D–J). The expressions of p-Akt, Akt, nuclear Nrf2, cytoplasm Nrf2 and HO-1 in the rats treated or untreated with DMSO, remifentanil and DEX was calculated by western blot. β-actin was the internal reference of p-Akt, Akt, cytoplasm Nrf2 and HO-1 in the rats treated or untreated with DMSO, remifentanil and DEX. H3 was the internal reference of nuclear Nrf2. * vs. Sham, * vs. CPB+DMSO; * or ** p < .05, *** or **** p < .01. CPB: cardiopulmonary bypass; DMSO: dimethyl sulfoxide; qRT-PCR: quantitative reverse transcription polymerase chain reaction; RPC: remifentanil; DEX: dexmedetomidine; p-Akt: phosphorylation of protein kinase B; Nrf2: NF-E2-related factor 2; HO-1: heme oxygenase-1.
CPB, suggesting that CPB does cause neuronal injury. Meanwhile, we found significant reductions in the levels of both S100β and NSE in the serum from CPB rats pretreated by remifentanil. Remifentanil preconditioning attenuated CPB-increased brain water content, lesions in the hippocampal CA1 area, brain nerve injury of rats. Cui et al. [16] demonstrated that remifentanil preconditioning induces neuronal activation in dorsal vagal complex in rat models of hepatic ischemia-reperfusion. From the results above, the preconditioning of remifentanil has been suggested to prevent against the brain injury in the rats with CPB.

In our study, plasma IL-10, and IL-6 levels were seen to significantly increase in the CPB group, and this increase was suppressed by preconditioning of remifentanil. It has also been reported that remifentanil attenuates increase in IL-6 mRNA level in the mouse brain and plasma [33]. In addition, oxidative stress plays a critical role in the development of neuroinflammation due to its activation in the CNS immune system, which results in a central inflammatory response that stimulates the production of some inflammatory factors [34]. It is indicated in the results of our study that CPB enhanced the level of MDA and reduced that of SOD, suggesting that CPB did induce oxidative stress in the brain tissue of rats, while the pretreatment of remifentanil could significantly prevent the CPB-induced increased level of MDA and reduced level of SOD, which is consistent with previous studies [19,20]. Data from a series of studies have reported that when POCD occurs, apoptosis is observed in the hippocampus, which serves an important role in the occurrence and development of POCD [35]. In fact, the results in our experiments showed that expressions of Bax and Cleaved caspase-3 were increased and that of Bcl-2 was decreased in the hippocampal tissues of rats after the CPB model was constructed, suggesting that CPB promoted the neuronal apoptosis. Similarly, we found that Remifentanil could prevent against CPB-induced changes in the expressions of apoptosis-related protein. The data above thus suggested that the neural-protective effects of the preconditioning using remifentanil were associated with the inhibition on the oxidative stress and apoptosis.

HO-1 is normally expressed at low levels in most of organs, whereas it is highly inducible in response to a variety of stimuli (such as hydrogen peroxide, UV irradiation, endotoxins, and hypoxia) to protect cells against oxidative and inflammatory injury [36]. Nrf2/HO-1 exerts its defense against early brain injury by abating oxidative stress, mitigating the inflammatory response, and preventing cortical cell death [35]. Hypoxia increases Nrf2-induced HO-1 expression via the PI3K/Akt pathway [37]. PI3K/Akt/Nrf2/HO-1 pathway mitigates oxidative stress, neurodegeneration, and memory impairment in a mouse model of Alzheimer’s disease [38]. The PI3K/Akt/Nrf2/HO-1 pathway is an important pathway in protection against epilepsy and seizure-induced brain injury under Dynorphin treatment through activation of κ-opioid receptor [39]. PI3K/Akt/Nrf2/HO-1 pathway participates in the regulation of H2O2-induced oxidative injury in endothelial cells and melanocytes [40,41]. The present study demonstrated that the preconditioning of remifentanil prevented the CPB-inhibited expressions of p-Akt, and HO-1, while it further promoted the effect of CPB in that of Nrf2. Besides, it was demonstrated in our study that the preconditioning with DEX (which is used as a neuroprotective drug in this study) had a similar effect to that of remifentanil. Therefore, the present study demonstrated that the preconditioning of remifentanil could prevent brain damage in CPB rats, and the mechanism may be related with activation of Akt/Nrf2 pathway.

In this study, we concluded that the preconditioning with remifentanil could prevent against the CBP-induced brain injury in rats, which is achieved by inhibiting the inflammation, oxidative stress and apoptosis via the Akt/Nrf2 signaling pathway in the neuron cells. These data may represent a new therapeutic application of the preconditioning with remifentanil in CBP-induced brain injury. However, the clinical applicability of these results remains to be additionally determined and further experimental exploration is needed.

**Ethical approval**

All animal experiments were conducted in accordance with the guidelines of the Chinese Council for Animal Care and Use, and the study was conducted at the First Affiliated Hospital of Guangxi Medical University. Our study has been ethically approved by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University (FAGMU201812357). All the researchers were dedicated to minimizing the discomfort of the animals throughout the experiment.
Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

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