Effects of PYRIN-containing Apaf1-like protein 1 on isoflurane-induced postoperative cognitive dysfunction in aged rats

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Abstract. Postoperative cognitive dysfunction (POCD) is a prevalent neurocognitive disorder following surgery and anesthesia, particularly in elderly patients. Isoflurane is a widely used anesthetic agent, which is associated with the development of POCD; however, the precise mechanisms remain unclear. In the present study, aged rats were exposed to 2% isoflurane to establish a POCD model. The expression of PYRIN-containing Apaf1-like protein 1 (PYPaF1) was knocked down using a lentivirus containing specific short hairpin RNA. Subsequently, the spatial learning ability of rats was assessed using the Morris water maze. In addition, mRNA and protein expression levels were detected using reverse transcription-quantitative PCR and western blot analysis, respectively. Immunofluorescence double staining was also used to determine the expression of PYPaF1 and Iba-1 in the hippocampus. Neural apoptosis was observed using TUNEL-NeuN double staining. The results revealed that isoflurane exposure impaired the spatial learning ability of rats, while PYPaF1 knockdown alleviated cognitive impairment. In addition, isoflurane exposure induced activation of the PYPaF1 inflammasome, as evidenced by elevated expression of PYPaF1 and apoptosis-associated speck-like protein containing a caspase recruitment domain, while silencing of PYPaF1 partially reversed this effect. Furthermore, isoflurane exposure promoted the activation of microglia and caspase-1, and the secretion of interleukin (IL)-1β and IL-18, all of which were alleviated following PYPaF1 silencing. Moreover, isoflurane exposure induced neuronal apoptosis, elevated the levels of Bax and cleaved caspase-3, and inhibited the expression of Bcl-2; all of these effects were partially abrogated following PYPaF1 silencing. In conclusion, the results of the present study indicated that PYPaF1 silencing partially abolished isoflurane-induced cognitive impairment, neuroinflammation and neuronal apoptosis. Therefore, PYPaF1 may be a potential therapeutic target for treatment of POCD.

Introduction

Postoperative cognitive dysfunction (POCD) is a type of postoperative complication that occurs in the central nervous system (CNS) (1,2). POCD is regarded as a mild neurocognitive disorder without a formal definition (3,4) and the diagnosis requires a validated neuropsychological test battery (5). POCD occurs in the first few weeks following surgery; it severely impairs cognitive function and increases mortality in patients who are affected (6). POCD is now considered to be a public health concern, which has a negative impact on the quality of life of patients who are affected and causes a high burden for social medical care (7). According to data from the Ministry of Civil Affairs of China, in 2018, the elderly population (>60 years old) reached 249.49 million, accounting for 17.9% of the total population of China (8). Thus, POCD may become a major health issue, due to lack of efficient diagnosis in the coming decades (9). Isoflurane-inhaled anesthesia was reported to be associated with the development of POCD by influencing brain activity, yet the exact mechanism has not been elucidated (10). Therefore, a deeper understanding of the role that isoflurane plays in the development of POCD is of great importance to discover novel approaches to eliminate isoflurane-induced cognitive impairment.

Increasing evidence has demonstrated that neuroinflammation is critical in the pathogenesis of neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease and POCD (11,12). Surgery and anesthesia have been reported to induce neuroinflammatory responses and promote the secretion of proinflammatory cytokines, such as interleukin (IL)-1β, IL-6 and tumor necrosis factor-α, which further activate microglia to an inflammatory phenotype (13,14). Microglia, innate immune cells of the brain, serve a crucial role in brain homeostasis. Activation of microglia is considered a negative event and it is involved in the development of neurocognitive disorders, including POCD (15,16). Furthermore, exposure to isoflurane has been shown to impair the spatial learning memory of aged mice, accompanied by activation of microglia and neuroinflammation (17). Yet the precise mechanisms remain unclear.
Inflammasomes are cytosolic protein complexes assembled by pattern recognition receptors (PRRs), pathogen-associated molecular patterns and danger-associated molecular patterns (18). To date, five inflammasomes have been discovered, among which the PYRIN-containing Apaf1-like protein 1 (PYPaF1) inflammasome is the most investigated (19). The PYPaF1 inflammasome is comprised of a sensor protein PYPaF1, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), aspartate-specific proteases and pro-caspase-1. PYPaF1 interacts with ASC, induces the activation of NF-κB and regulates inflammatory-associated processes (20). The PYPaF1 inflammasome activates the protease caspase-1, promotes the secretion of IL-1β and IL-18, and further induces neuroinflammation (21). Studies have shown that PYPaF1 may contribute to neuroinflammation in numerous cognitive impairment-related diseases (19), and inhibition of PYPaF1 activation has been found to inhibit inflammation in the brain (22); however, to the best of our knowledge, its role in POCD has not been elucidated.

In our preliminary studies, PYPaF1 was upregulated in the hippocampus of POCD rats. As PYPaF1 is associated with inflammation, it was hypothesized that PYPaF1 may have an important role in the development of POCD. The aim of the present study was to determine the possible mechanisms of POCD by examining PYPaF1 and inflammation, using a rat POCD model via isoflurane exposure. In addition, the role that PYPaF1 plays in microglial activation and neuroinflammation was investigated.

Materials and methods

Rat POCD model. A total of 138 healthy 20-month-old male Sprague Dawley (SD) rats (weight, 500-520 g; Liaoning Changsheng Biotechnology Co. Ltd.) were randomly divided into four groups: Control; isoflurane; isoflurane + short hairpin (sh)RNA-PYPaF1; and isoflurane + shRNA-negative control (NC). Rats were maintained in a standard experimental rodent room under a 12-h light/dark circle, at a temperature of 23±1°C and relative humidity of 50±1%, with free access to water and food. The animals were adaptively fed for 1 week before establishment of the animal model. For the isoflurane + shRNA-PYPaF1 group, rats were injected with 5 µl lentivirus containing PYPaF1-shRNA [10⁶ transduction units (TU)/ml] into the right lateral ventricle; for the isoflurane + shRNA-NC group, rats were injected with 5 µl lentivirus carrying NC-shRNA [10⁶ TU/ml] into the right lateral ventricle. A total of 3 days after lentiviral injection, rats in the isoflurane, isoflurane + shRNA-PYPaF1 and isoflurane + shRNA-NC groups were maintained in a chamber prefilled with 2% isoflurane in 100% oxygen for 4 h, rats in the control group were given 100% oxygen for 4 h (gas flow rate, 2 l/min) (23). A total of 18 rats in each group were randomly selected and euthanized by intraperitoneal injection of 200 mg/kg sodium pentobarbital on day 3, and six rats in each group were euthanized on day 7 following isoflurane exposure, and the hippocampi were collected. Hippocampi from 12 rats euthanized on day 3 and six rats euthanized on day 7 were cryopreserved in liquid nitrogen for molecular biological analysis. Hippocampi from six rats euthanized on day 7 were fixed with 4% paraformaldehyde at 4°C overnight for histological examinations. The other six rats in each group were used for behavioral testing. Another 18 SD rats were randomly divided into three groups (control, shRNA-PYPaF1 or shRNA-NC) for the assessment of transfection efficiency. All animal experiments were performed at the animal center at the First Hospital of Jilin University (Changchun, China) following the guidelines for the care and use of laboratory animals (24), and this study was approved by the First Hospital of Jilin University.

Construction of PYPaF1 shRNA lentiviral vectors. To generate PYPaF1-targeting shRNA expressing plasmids, double-stranded oligonucleotides encoding PYPaF1 shRNA were amplified in a system containing 5X oligo annealing buffer (1 mM EDTA, 50 mM NaCl, 10 mM Tris), sense and antisense primers containing the PYPaF1 shRNA sequence (Sangon Biotech Co., Ltd.) using the following reaction conditions: 95°C for 5 min, 90°C for 5 min, 85°C for 5 min and 80°C for 5 min, followed by overnight annealing at room temperature. The primer sequences were as follows: Forward, 5'-ccgggcccCGCTATGTACTATCTGCTATccagagaTAGCAGATAGTACATAGCCTTtttt-3' and reverse, 5'-aataaaaaGCGTATGACTCTGCTACTctcttgggTAGCAGATAGTACATAGCCTggg-3'. After the Tet-pLKO-puro lentivector (cat. no. 21915; Addgene, Inc.) was linearized with FastDigest AgeI and FastDigeTest EcoRI (cat. nos. FD1646 and FD0274; Thermo Fisher Scientific, Inc.), the oligonucleotides encoding PYPaF1 shRNA were inserted into the lentivector with T4 DNA Ligase (cat. no. 2011A; Takara Biotechnology Co., Ltd.). The recombinant plasmids were then transformed into Escherichia coli DH5α-competent cells (cat. no. 9057; Takara Biotechnology Co., Ltd.). The plasmids were extracted from the positive clones using a Plasmid Maxi Preparation kit (cat. no. DP2802; BioTeke Corporation). A total of 24 h after transfection, 293T cells were plated into a 10-cm plate at density of 6x10⁶. Tet-pLKO-puro-PYPaF1 shRNA lentiviral vectors (3 µg), packaging plasmid pSIN2X (5 µg; cat. no. 12260; Addgene, Inc.) and envelop plasmid pMD2.G (5 µg; cat. no. 12259; Addgene, Inc.) were co-transfected into 293T cells (procell Life Science & Technology Co., Ltd.) using Lipofectamine® 2000 (cat. no. L3000-008; Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 48 h to perform viral packaging. Lentivirus-containing supernatants were then centrifuged at 4,000 x g at 4°C for 10 min, filtered through a 0.45-µm filter and centrifuged at 4,000 x g at 4°C for 5 min. The lentivirus stock was collected and stored at -80°C. The shRNA sequences were as follows: PYPaF1 shRNA, sense, 5'-CCGGCCCGGCCGTAGTACTATCTGCTATccagagaTAGCAGATAGTACATAGCCTTtttt-3' and antisense, 5'-AATTTAAAAGGGCATGTAGTACTCTTGGATAGCAGATAGTACATAGCCC GG-3'; and NC-shRNA, sense, 5'-CCGGCCCGGCCGTAGTACTATCTGCTATccagagaTAGCAGATAGTACATAGCCTTtttt-3' and antisense, 5'-AATTTAAAAGGGCATGTAGTACTCTTGGATAGCAGATAGTACATAGCCC GG-3'.

Reverse transcription-quantitative PCR (RT-qPCR). Hippocampal tissues were lysed and total RNA was extracted using commercial RNAprep pure kit (Tiangen Biotech Co., Ltd.). Total RNA was subsequently reverse transcribed into cDNA using oligo(dT)₁₂, dNTP, M-MLV and RNase inhibitor
Western blot analysis. Hippocampal tissues were lysed with RIPA buffer and protein concentration was determined using a BCA protein quantification kit (both purchased from Beijing Solarbio Science and Technology Co., Ltd.). Proteins (40 µg) were separated by SDS-PAGE using 8 and 14% gels and transferred to PVDF membranes (EMD Millipore; Merck KGaA). After blocking with 5% skimmed milk for 1 h at room temperature, PVDF membranes were incubated with primary antibodies at 4°C overnight, followed by incubation with secondary antibodies at 37°C for 1 h. Subsequently, the protein bands were visualized using ECL reagent (Beijing Solarbio Science and Technology Co., Ltd.) and GAPDH antibody (1:10,000; cat. no. A0516; Beyotime Institute of Biotechnology). Tissue sections were washed and incubated at 4°C overnight with FITC-labeled goat anti-rabbit IgG (1:200; cat. no. A0568; Beyotime Institute of Biotechnology) and Cy3 labeled goat anti-rabbit IgG (1:200; cat. no. A0516; Beyotime Institute of Biotechnology) for 90 min at room temperature, and incubated with DAPI at room temperature for 5 min (Beyotime Institute of Biotechnology). Tissue sections were sealed with anti-fluorescence quenching reagent (Beijing Solarbio Science and Technology Co., Ltd.) and images were obtained using a fluorescence microscope (x400, magnification).

**TUNEL-NeuN staining.** Hippocampal sections were deparaffinized and incubated with 0.1% Triton X-100 for 8 min at room temperature (Beyotime Institute of Biotechnology). After retrieval with citric acid/sodium citrate solution at 95°C for 10 min, tissues were incubated with TUNEL solution (enzyme solution:label solution, 1:9; Roche Diagnostics) for 60 min at 37°C, then with NeuN antibody (1:300; cat. no. ab104224; Abcam) at 4°C overnight. Samples were then washed with PBS three times and incubated with Cy3 labeled goat anti-mouse IgG (1:200; cat. no. A0521; Beyotime Institute of Biotechnology) for 60 min at room temperature. Tissues were washed and incubated with Cy3 labeled goat anti-rabbit IgG (1:200; cat. no. A0516; Beyotime Institute of Biotechnology) for 90 min at room temperature, and incubated with DAPI at room temperature for 5 min (Beyotime Institute of Biotechnology). Tissue sections were sealed with anti-fluorescence quenching reagent (Beijing Solarbio Science and Technology Co., Ltd.) and images were obtained using a fluorescence microscope (x400, magnification). Three sections from each sample and three fields of view in each section were randomly selected for observation, and the average values were used as the final data.

**Morris water maze.** The Morris water maze was performed 7 days after isoflurane exposure to assess the spatial learning and memory ability of rats. The water maze was composed of a circular tub (diameter, 180 cm) and a colorless escape platform (diameter, 12 cm) submerged 2 cm below the water surface. Rats were placed into the water from different quadrants up against the wall, and the time it took to escape was recorded. If rats failed to identify the platform within 120 sec, they were guided to the platform and allowed to rest for 15 sec. Rats were subjected to the training from different quadrants four times a day, for 5 days. Subsequently, the platform was removed on the 6th day of the trial, and the escape time, times of platform crossing and retention time at the target quadrant were recorded.

**ELISA.** Rat hippocampal tissues were homogenized on ice in saline to extract protein and protein concentration was quantified using a BCA protein quantification kit (Beijing Solarbio Science and Technology Co., Ltd.). The amount of IL-1β and IL-18 was determined using a commercial IL-1β ELISA kit [cat. no. 70-EK301B/3-96; MultiSciences (Lianke) Biotech Co., Ltd.] and IL-18 ELISA kit (cat. no. SEA064Ra; Wuhan USCN Business Co., Ltd.), according to the manufacturers' instructions.

**Caspase-1 activity detection.** Hippocampal tissue (10 mg) was lysed in 100 µl lysis buffer and protein concentration was quantified using a Bradford protein assay kit (Beyotime Institute of Biotechnology). Caspase-1 activity was determined by measuring the optical density values at 405 nm using a commercial caspase-1 activity assay kit (cat. no. BC1810; Beijing Solarbio Science and Technology Co., Ltd.), according to the manufacturer's protocol.
Results

**PYP1 expression is successfully silenced by shRNA-PYP1.** The expression of PYP1 in the hippocampus was detected 3 days after lentiviral infection to confirm the efficiency of RNA interference (RNAi). The mRNA expression levels of PYP1 in the hippocampus were measured using reverse transcription-quantitative PCR and western blot analysis, respectively. "***P<0.001. NC, negative control; PYP1, PYrin-containing Apaf1-like protein 1; shRNA, short hairpin RNA.

**PYP1 silencing alleviates the cognitive dysfunction induced by isoflurane in rats.** The Morris water maze was performed to evaluate the spatial learning ability of rats 7 days after isoflurane treatment. Escape time of rats in the isoflurane group was significantly increased compared with the control group throughout the experiment (P<0.001). shRNA-NC did not apparently change the escape time of isoflurane-exposed rats. However, PYP1-targeting shRNA markedly shortened the escape time of isoflurane-exposed rats compared with the isoflurane + shRNA-NC group from day 3 (P<0.05 at day 3; P<0.01 at days 4 and 5; Fig. 2A). Times of platform crossing and retention time at the target quadrant were recorded on day 6 of the Morris water maze test. Rats subjected to isoflurane exposure had a lower number of platform crossings compared with rats in the control group, while PYP1 silencing increased the number of platform crossings (Fig. 2B). Furthermore, rats exposed to isoflurane spent less time at the target quadrant compared with rats in the control group, while silencing of PYP1 increased the retention time (Fig. 2C).

**PYP1 silencing inhibits the activation of microglia.**
The mRNA expression levels of PYPAF1 were significantly higher in rats exposed to isoflurane compared with the control group, while PYPAF1 silencing using a lentivirus decreased the levels of PYPAF1 on day 3. Similarly, significant associations were observed on day 7 following isoflurane treatment (Fig. 3A). Isoflurane treatment significantly increased the protein expression levels of PYPAF1 and ASC on day 3, whereas PYPAF1 silencing partially abrogated the increase and the trend was more notable on day 7 following isoflurane exposure (Fig. 3B and C). Furthermore, the immunofluorescence assay revealed that isoflurane exposure increased the expression of Iba-1, which was significantly alleviated by knockdown of PYPAF1 (Fig. 3D).

**PYPAF1 silencing inhibits neuroinflammation.** RT-qPCR and ELISA were performed to determine the levels of IL-1β and IL-18. The mRNA expression and concentration of IL-1β and IL-18 in the hippocampus were increased following isoflurane exposure, whereas PYPAF1 silencing decreased their levels (Fig. 4A and B). Furthermore, isoflurane treatment enhanced the activity of caspase-1, which was partially abolished by silencing PYPAF1 (Fig. 4C). These results indicated that silencing of PYPAF1 inhibited neuroinflammation in rats.

**PYPAF1 silencing alleviates neuronal apoptosis.** TUNEL assay was performed to assess neuronal apoptosis in the hippocampus. Apoptotic neurons were markedly increased...
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Following isoflurane exposure, which was partially reversed following PYPAF1 knockdown. Isoflurane treatment decreased the expression of NeuN, whereas silencing PYPAF1 alleviated the effects of isoflurane (Fig. 5A). Furthermore, the protein expression levels of Bax and cleaved caspase-3 were increased by isoflurane, and the elevation was partially abolished by silencing of PYPAF1. The protein levels of Bcl-2 revealed the opposite results (Fig. 5B).

Figure 4. PYPAF1 knockdown inhibits the activity of caspase-1, and expression of IL-1β and IL-18. (A) mRNA levels and (B) concentration of IL-1β and IL-18 in the hippocampus was determined using reverse transcription-quantitative PCR and ELISA, respectively, on day 3 following isoflurane treatment. (C) Activity of caspase-1 in the hippocampus was assessed using a commercial caspase-1 activity assay kit on day 3 following isoflurane treatment. ***P<0.001. IL, interleukin; NC, negative control; PYPAF1, PYrin-containing a paf1-like protein 1; shRNA, short hairpin RNA.

Figure 5. PYPAF1 silencing alleviates neuronal apoptosis. (A) Neuronal apoptosis in the hippocampus was determined using TUNEL-NeuN double staining on day 3 following isoflurane treatment. (B) Protein expression levels of Bcl-2, Bax and cleaved caspase-3 in the hippocampus were determined using western blot analysis. ***P<0.001. Iso, isoflurane; NC, negative control; PYPAF1, PYRIN-containing Apaf1-like protein 1; shRNA, short hairpin RNA.
Discussion

In the present study, isoflurane upregulated the expression levels of PYPAF1 in the hippocampus of aged rats. RNAi-mediated PYPAF1 knockdown significantly improved learning and memory function of isoflurane-exposed aged rats. In addition, PYPAF1 knockdown inhibited activation of microglia, production of proinflammatory cytokines and neuronal apoptosis in the hippocampus of isoflurane-exposed aged rats.

Isoflurane is a widely used inhalational anesthetic agent owing to its low cost and relatively few side effects (26). Inhaled anesthesia could induce cerebral cognitive alterations in elderly patients (10,27). Furthermore, a clinical trial involving elderly patients that were subjected to laparoscopic cholecystectomy revealed that patients who received isoflurane anesthesia displayed a significantly higher incidence of POCD compared with those who received sevoflurane and propofol (10,28). In the present study, the spatial learning ability of rats exposed to isoflurane was markedly reduced, which was accompanied by increased expression of PYPAF1 and ASC, whereas shRNA-mediated PYPAF1 knockdown markedly alleviated the cognitive dysfunction of aged rats and decreased the expression of ASC. The PYPAF1 inflammasome is abundantly expressed in the CNS and activation of the PYPAF1 inflammasome is involved in the pathogenesis of neurological diseases, including AD and depression (29,30). PYPAF1 is the cytosolic sensor molecule of the PYPAF1 inflammasome and abundant expression of PYPAF1 is crucial for inflammasome formation (21). ASC activates the PYPAF1 inflammasome by recruiting the cysteine protease pro-caspase-1 via its caspase recruitment domain (31). In the present study, shRNA-mediated PYPAF1 knockdown hindered the assembling of the PYPAF1 inflammasome and suppressed the expression of ASC. Furthermore, isoflurane exposure led to the activation of caspase-1, which was also inhibited following knockdown of PYPAF1. PYPAF1 silencing also abolished isoflurane-induced elevated IL-1β and IL-18 at both the mRNA and protein levels. These findings are consistent with previous reports, which indicated that activation of the PYPAF1 inflammasome further activated inflammatory protease caspase-1, and promoted the secretion of pro-inflammatory cytokines IL-1β and IL-18 (21,32). Increased levels of IL-1β and IL-18 may trigger a series of signaling pathways and inflammatory responses, which could lead to neuronal injury or apoptosis, whereas knockdown of IL-1β in mice significantly alleviated neuroinflammatory and memory dysfunction following surgery and anesthesia (33,34). The results from the present study indicated that the PYPAF1/ASC pathway may contribute to isoflurane-induced POCD in aged rats.

Microglia, a primary effector cell that responds to pathological challenges in the CNS, serves a crucial role in neuroprotection and in the maintenance of hemostasis (35). Mild activation of microglia exerts a protective effect on the CNS, whereas its over-activation results in neuronal dysfunction (36,37). Furthermore, microglia express numerous PRRs that are essential for assembling the inflammasome (38). In the present study, isoflurane exposure led to activation of microglia in the hippocampus of aged rats, and PYPAF1 knockdown inhibited the activation of microglia, as evidenced by the decrease in the expression of Iba-1. It is well known that microglial activation occurs in the early stage of neuroinflammation, which leads to the production of proinflammatory cytokines and induces cognitive dysfunction (39). In addition, aging causes microglia to be more susceptible to activation (40). The findings from the present study indicated that PYPAF1 may have a critical role in microglial activation following isoflurane exposure. However, the present study has not revealed whether this inhibitory effect on microglial activation is direct or indirect; therefore, further investigation is required in future studies.

Neuronal function is the foundation of learning and memory (41). In the present study, isoflurane exposure induced neuronal apoptosis and decreased NeuN expression in the rat hippocampus, whereas PYPAF1 silencing alleviated neuronal apoptosis and elevated the levels of NeuN. Isoflurane exposure elevated the expression of pro-apoptotic factor, Bax, and cleaved caspase-3, and decreased the expression of the anti-apoptotic factor, Bcl-2. Furthermore, silencing of PYPAF1 abrogated isoflurane-induced neuronal apoptosis. These results suggested that PYPAF1 knockdown may inhibit neuronal apoptosis in the hippocampus of aged rats exposed to isoflurane. However, whether PYPAF1-targeting shRNA directly affects apoptotic pathways, or inhibits apoptosis acts via its effect on inflammasome inhibition, requires further investigation. The role that the PYPAF1 inflammasome plays in the development of POCD has not been well reported. Wang et al (42) revealed that isoflurane exposure induced cognitive impairment and neuroinflammation, which are more likely to occur in aged mice, and a caspase-1 inhibitor mitigated cognitive dysfunction. In addition, knockdown of PYPAF1 in BV-2 cells inhibited PYPAF1 inflammasome activation and IL-1β production (42). To the best of our knowledge, the present study was the first to reveal that PYPAF1 knockdown protects aged rats from isoflurane-induced cognitive impairment, which may be associated with the effect on inhibition of microglial activation and neuronal apoptosis in the hippocampus.

There are limitations to the present study. Firstly, analysis of the hippocampus and proinflammatory factors were examined 3 and 7 days following exposure to isoflurane. The changes in PYPAF1 expression at earlier and later time points will be observed in future studies. Secondly, the exact role of PYPAF1 in different types of brain cells, such as microglia and neurons, under the exposure of isoflurane was not determined, as these could not be isolated and cultured from aged animals.

In conclusion, the findings from the present study demonstrated that PYPAF1 knockdown alleviated isoflurane-induced cognitive impairment in aged rats. Neuroinflammation and neuronal apoptosis inhibition may contribute to this effect. Therefore, the PYPAF1 inflammasome may serve as a potential therapeutic target for the treatment of POCD.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
YZ designed the study. XZ, XF, FL and JQ contributed to the acquisition, analysis and interpretation of data. XZ drafted the article. YZ critically revised it for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate
All animal experiments were performed at the animal center at the First Hospital of Jilin University following the guidelines for the care and use of laboratory animals, and the study was approved by the First Hospital of Jilin University.

Patient consent for publication
Not applicable.

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

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