

# Ketamine administration ameliorates anesthesia and surgery-induced cognitive dysfunction via activation of TRPV4 channel opening

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**Abstract.** Perioperative neurocognitive disorder (PND) is a common complication associated with anesthesia and surgery in the elderly. The dysfunction of transient receptor potential vanilloid 4 (TRPV4) has been associated with a number of diseases, including Alzheimer's disease. Given that ketamine can reportedly improve PNDs, the present study sought to determine whether ketamine-induced PND alleviation was mediated by activation of TRPV4 channel opening. A total of 120, 20-month-old male C57BL/6 mice were randomly divided into five groups: Vehicle, PND (tibial fracture surgery), PND + ketamine (Ket), PND + Ket + HC-067047 (HC), and PND + HC groups. Ketamine (0.5 mg/kg) was administered intraperitoneally once a day for 3 days after surgery and HC-067047 (1  $\mu$ mol/2  $\mu$ l), an antagonist of TRPV4, was administered via the left lateral ventricle 30 min before ketamine treatment. Superoxide dismutase (SOD), malondialdehyde (MDA), lipid peroxidation (LPO), IL-1 $\beta$ , IL-6, adenosine monophosphate-activated protein kinase (AMPK), NF- $\kappa$ B, TNF- $\alpha$  and IFN- $\beta$  levels were determined 3 days after surgery. At 28 days after surgery, fear conditioning and novel object recognition were assessed, and A $\beta$ 1-42 levels were measured and ionized calcium binding adaptor molecule 1 (Iba1) staining was conducted on day 31 after surgery. The results revealed that ketamine administration upregulated total SOD activity, downregulated MDA and LPO content, mitigated phosphorylated (p)-NF- $\kappa$ B, TNF- $\alpha$  mRNA and IFN- $\beta$  mRNA expression in the hippocampus, and promoted p-AMPK 3 days after surgery. Furthermore, it was found that ketamine increased both context- and tone-dependent fear conditioning, and the time spent exploring a novel object, and reduced A $\beta$  peptide levels and microglial activation 30 days

after surgery. Notably, these changes could be reversed by HC-067047 to a certain extent. In conclusion, ketamine improved PND in aged mice after tibial fracture surgery and the potential mechanism may involve activation of the TRPV4/AMPK/NF- $\kappa$ B signaling pathway.

## Introduction

Postoperative cognitive dysfunction (POCD) is a complication of anesthesia and surgery prevalent in the elderly, which is characterized by memory loss and cognitive deficiencies (1,2). A study recommended that the clinical terminology for cognitive dysfunction temporally relative to surgery and/or anesthesia should be modified from POCD to perioperative neurocognitive disorder (PND) (2). PNDs are usually associated with a decrease in the quality of life in elderly patients (3,4). As with other neurodegenerative diseases, including Alzheimer's, the precise mechanisms and therapies of PND should be further investigated.

In clinical practice, ketamine has been used to induce short-term anesthesia and analgesia via inhibiting N-methyl-D aspartic acid (NMDA) receptors (5). Notably, the use of ketamine in other domains, including managing acute and chronic pain, and as an antidepressant for mental disorders, has caused widespread concern (6,7). Ketamine exerts neuroprotective effects by decreasing the systemic production of inflammatory factors induced by surgery (8) and attenuates cognitive dysfunction in patients undergoing cardiac surgery with a concomitant anti-inflammatory effect (9). Until now, the mechanisms underlying the efficiency of ketamine in treating cognitive dysfunction have not been extensively studied.

The transient receptor potential vanilloid 4 (TRPV4) channel, which belongs to the mammalian transient receptor potential superfamily of cation channels, has been reported to be widely distributed in neurons and glial cells (10,11). As a cell surface-expressed, non-selective cation channel, TRPV4 can be activated by chemical, mechanical and osmotic stimuli via regulation of calcium ion influx (12). Notably, the inhibition of TRPV4 channel opening reportedly contributes to neuronal death and inflammatory response in a model of intracerebral hemorrhage (13), while activation of TRPV4 channel opening can participate in the regulation of neuronal excitability and behavior in mammals (14). Most importantly, it has been reported that silencing TRPV4 can block excessive

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ketamine-induced neurotoxic effects (15). Based on these findings, the present study explored whether the opening of TRPV4 channels mediated ketamine-induced neuroprotection against PND.

The present study established a tibial fracture model and then investigated whether ketamine administration ameliorated PND in aged mice after surgical treatment. In addition, the role of TRPV4 channels in mediating the neuroprotective effects of ketamine was determined.

## Materials and methods

**Experimental animals.** A total of 120, 20-month-old adult male C57BL/6 mice (weight, 35–40 g) were purchased from Changsheng Biotechnology Co., Ltd. As per a previous study, 20-month-old mice were considered as aged mice (16). All mice were allowed free access to food and water, and kept in a 12-h light/dark cycle facility at 25±1°C (humidity, 50–70%). All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (17). The present study was approved by the Animal Review Board of The Second Affiliated Hospital of Jiaying University (JXEY-2021JX122) (Jiaying, China). In addition, all experiments complied with the Animal Research: Reporting *In Vivo* Experiments (ARRIVE) guidelines (18).

**Group assignment.** Male C57/BL mice were divided into one of the following five groups according to a computer-based randomization method (n=24/group): i) Vehicle; ii) PND; iii) PND + ketamine (Ket); iv) PND + Ket + HC-067047 (HC); v) PND + HC. Mice in the PND groups were subjected to tibial fracture surgery as described in previous studies (19,20). Ketamine administration (0.5 mg/kg; Gutian Fuxing Pharmaceutical Co., Ltd.) was performed once a day for 3 days (the first injection from 2 h after surgical treatment) as indicated by previous studies (20,21) and our preliminary experiment. 2 µl HC-067047 (1 µmol, cat. no. HY-100208; MedChemExpress), a selective antagonist of TRPV4, was dissolved in 10% dimethyl sulfoxide (DMSO; cat. no. D8418; Beyotime Institute of Biotechnology) and 90% corn oil (cat. no. HY-Y1888; MedChemExpress) and injected into the left lateral ventricle at 30 min before ketamine administration. The co-ordinates of the left lateral ventricle located using a stereotaxic instrument were as follows: 0.3 mm posterior to the bregma, 1.0 mm lateral to the midline and 2.5 mm below the skull. Vehicle (2 µl) for HC-067047 was administered with an equivalent volume of DMSO plus corn oil as a control to all groups not treated with HC-067047. If mice lost their appetite for 5 days or stopped drinking for 3 days, lost >20% of their body weight, and were unable to eat and drink before the scheduled experimental endpoint, they were sacrificed. After mice were anesthetized with 7–8% sevoflurane for 5–7 min, reflexes disappeared. When the monitor indicated cardiac arrest, respiratory arrest and pupil dilation, the mice were euthanized by cervical dislocation. A total of 60 mice (n=12/group) were sampled 3 days after surgery to assess oxidative stress (n=6) and inflammatory response (n=6) in the five groups. A total of 60 mice (n=12/group) were assigned to behavioral tests 28 days after surgery and sacrificed at day 31 for Aβ1-42 analysis (n=6) and immunofluorescence (n=6)

staining. The timeline of the experimental procedure is shown in Fig. 1.

**Tibial fracture surgery.** Tibial fracture surgery was used to stimulate PND in mice, as described by previous studies (22,23). Mice were subjected to 7–8% sevoflurane in self-made inhalation anesthesia boxes as anesthesia induction. Mice were then anesthetized with 3–4% sevoflurane with a face mask for anesthesia maintenance and kept warm at 37–38°C with a warming pad; a heart rate between 100 and 120 bpm was maintained. Tibial fracture surgery was performed as follows: i) The left hind paw was shaved and disinfected; ii) a 0.38-mm pin was inserted in the intramedullary canal after making a median incision on the left hind paw; iii) the periosteum was stripped with a periosteal elevator; iv) the osteotomy was performed with scissors; v) the median incision was sutured and disinfected; vi) the incision was blocked by 1.0% ropivacaine (0.1 ml) for postoperative analgesia. Under anesthesia, a skin incision, suture and incision block on the left hind paw were performed in mice of the vehicle group.

**Novel object recognition.** The cognitive ability of mice was assessed via a novel object recognition test (24). At days 28 and 29, after the tibial fracture surgery, mice were placed in a 60x60x40-cm box with black walls for 5 min/day. On day 30 after surgery, mice were exposed to two identical cubic objects placed in the right and left corners of the box until they had explored for a total of 5 min. During the test phase, the right cubic object was replaced by a novel spherical object; subsequently, the mice were allowed to probe the two objects for 5 min. A video analysis system provided by Shanghai Jiliang Software Science & Technology Co., Ltd. was used to analyze the trajectory of mice. Cognitive ability was assessed using recognition index (RI), which was calculated as follows:  $RI = \frac{\text{novel object exploration time}}{\text{novel object exploration time} + \text{familiar object exploration time}}$ .

**Fear conditioning.** On day 28 after surgery, mice were assigned to a fear conditioning test. The mice were kept in a dark chamber wiped with 70% alcohol and subjected to three tone-foot shock pairings (tone: 2,000 Hz; 80 dB; 60 sec; foot shock: 1 mA; 2 sec). After the final foot shock, the animals were kept in the chamber for 60 sec and then returned to the home cage. The next day, the mice were exposed to the same chamber without tone-foot shock pairings for 3 min. After 2 h, the mice were placed in a novel chamber with a different context (transparent walls and light) and smell (wiped with 1% acetic acid) from the first test chamber. The freezing behaviors with and without the tone stimulus were recorded for 3 min using a video analysis system (XR-XZ301; Shanghai XinRuan Information Technology Co., Ltd.). The freezing behaviors, including 3 min in the dark chamber (context-related) and 3 min in the transparent chamber (tone-related), were used to assess the rodent's memory.

**Amyloid β1-42, IL-1β and IL-6 ELISA.** The mice were deeply anesthetized with 8% sevoflurane and perfused with heparin saline via the aorta. The hippocampal tissues for ELISA, reverse transcription-quantitative (RT-q) PCR and western blot analysis were isolated and homogenized. Total protein

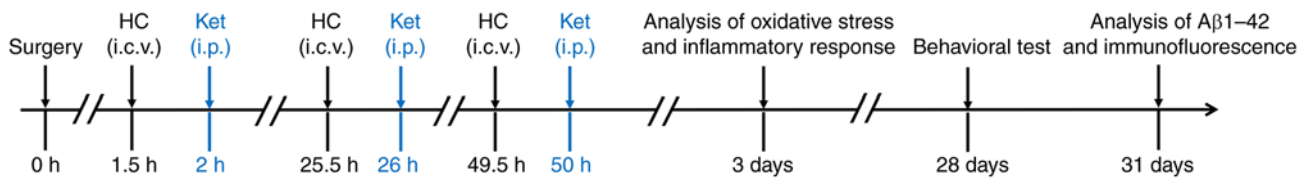


Figure 1. Experimental schematic diagram. Mice underwent tibial fracture surgery to stimulate perioperative neurocognitive disorder. Ket (0.5 mg/kg, i.p.) was administered once a day for 3 days after surgical exposure; HC (1  $\mu$ mol/2  $\mu$ l), an antagonist of transient receptor potential vanilloid 4, was administered via the left lateral ventricle 30 min before Ket treatment. Novel object recognition and fear conditioning tests were used to evaluate cognitive dysfunction. Ket, ketamine; HC, HC-067047; i.c.v., intracerebroventricular; i.p., intraperitoneal.

was extracted using cell lysis buffer (cat. no. P0013, Beyotime Institute of Biotechnology) and quantified by BCA assay. Based on the manufacturer's instructions, the levels of A $\beta$ 1-42 (cat. no. CS-ELISA2397; Chuntest Biotechnology Co., Ltd.), IL-1 $\beta$  (cat. no. PI301; Beyotime Institute of Biotechnology) and IL-6 (cat. no. EK0411; Wuhan Boster Biological Technology, Ltd.) were analyzed using sandwich ELISA kits.

**Immunofluorescence.** Mice were anesthetized with 8% sevoflurane, and were then perfused with heparin saline and 4% paraformaldehyde via the aorta. Following fixing with 4% paraformaldehyde at room temperature for 24 h, the cerebral tissue containing the hippocampus was dehydrated with 50-90% ethanol at room temperature, then the cerebral tissue was permeabilized in 50% xylene-ethanol at room temperature for 30 min then in 50% xylene-paraffin at 60°C for 15 min and embedded in paraffin. Subsequently, 4- $\mu$ m paraffin-embedded sections were dewaxed and rehydrated with alcohol, incubated with 0.1% Triton X-100 for 30 min at room temperature (cat. no. T8200; Beijing Solarbio Science & Technology Co., Ltd.) and sealed with 5% standard bovine serum (cat. no. A8010; Beijing Solarbio Science & Technology Co., Ltd.) for 1 h at room temperature. After rinsing with PBS, a polyclonal goat anti-ionized calcium binding adaptor molecule 1 (Iba1) primary antibody (cat. no. ab5076; 1:400; Abcam) was used to incubate the sections at 4°C overnight. The secondary antibody (Cy3-conjugated donkey anti-goat IgG; cat. no. A0502; 1:1,000; Beyotime Institute of Biotechnology) was then used to incubate the sections for 1 h after washing three times with PBS. 4',6-diamidino-2-phenylindole (DAPI; 10  $\mu$ g/ml, cat. no. C1002; Beyotime Institute of Biotechnology) was used to label nuclei. Fluorescence images were captured by a fluorescence microscope (DP70; Olympus Corporation), while the intensity and number were analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Inc.).

**Measurement of total superoxide dismutase (SOD), malondialdehyde (MDA) and lipid peroxidation (LPO) content.** At 3 days following surgery, the total SOD activity, and MDA and lipid LPO levels in the hippocampus were assessed using a SOD assay kit (WST-8 method; cat. no. S0103; Beyotime Institute of Biotechnology), an MDA assay kit (cat. no. S0131; Beyotime Institute of Biotechnology) and an LPO kit (cat. no. JLC13854, Gelatins; Jiang Lanchun), respectively, according to the manufacturers' instructions.

**Reverse transcription-quantitative (RT-q) PCR.** At 3 days following surgery, total RNA was extracted from

hippocampal tissues with Beyozol (cat. no. R0011; Beyotime Institute of Biotechnology) and then reverse transcribed into cDNA using the BeyoRT First Strand cDNA Synthesis kit (cat. no. D7166; Beyotime Institute of Biotechnology) according to the manufacturers' protocols. The RT-qPCR system (Step One; Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to assess the reaction mixtures, which contained: 1  $\mu$ l cDNA, 0.2  $\mu$ l forward primer, 0.2  $\mu$ l reverse primer, 5  $\mu$ l BeyoFast SYBR Green qPCR Mix (cat. no. D7260; Beyotime Institute of Biotechnology) and 3.6  $\mu$ l nuclease-free water. The PCR amplification conditions were as follows: 95°C for 10 min and 45 cycles of 95°C for 10 sec and 60°C for 1 min. The mRNA expression levels were normalized to  $\beta$ -actin and calculated with the  $2^{-\Delta\Delta C_q}$  method (25). The sequence of primers used for qPCR were: TNF- $\alpha$ , forward (F) 5'-GACCCTCACACTCAGATCATCTTCT-3' and reverse (R) 5'-CCTCCACTTGGTGGTTTGGCT-3'; IFN- $\beta$ , F 5'-GCCCTCTCCATCGACTACAAG-3' and R 5'-AAGACATTCTGGAGCATCACTTG-3';  $\beta$ -actin, F 5'-TTTGCAGCTCCTTCGTTGC-3' and R 5'-TCGTCA TCCATGGCGAACT-3'.

**Western blotting.** At 3 days following surgery, total protein was extracted using cell lysis buffer (cat. no. P0013, Beyotime Institute of Biotechnology) and quantified by BCA assay. Protein samples (30  $\mu$ g) from hippocampal tissues were mixed with loading buffer (cat. no. P0015; Beyotime Institute of Biotechnology), boiled, separated by SDS-PAGE (12% gel) and transferred to a PVDF membrane. Skimmed milk (5%) was used to block the PVDF membrane for 2 h at 25°C. After washing three times with TBS-0.05% Tween (5 min/wash), the PVDF membrane was incubated with the following primary antibodies: Monoclonal rabbit anti-phosphorylated (p)-adenosine monophosphate-activated protein kinase (AMPK) (Ser496; cat. no. AF2677; 1:500), monoclonal total AMPK (cat. no. AF1627; 1:500), polyclonal p-NF- $\kappa$ B p65 (cat. no. AF5875; 1:500) and polyclonal NF- $\kappa$ B p65 (cat. no. AF0246; 1:1,000) (all from Beyotime Institute of Biotechnology) at 4°C overnight. The next day, a secondary antibody (HRP-labeled Goat Anti-Rabbit IgG; cat. no. A0208, 1:2,000; Beyotime Institute of Biotechnology) was used to incubate the PVDF membrane at 25°C for 1 h. Following incubation with BeyoECL Plus (cat. no. P0018; Beyotime Institute of Biotechnology) for 5 min at 25°C, protein bands were analyzed using Image Lab software (version 6.0; Bio-Rad Laboratories, Inc.). GAPDH (1:1,000; cat. no. K106389P; Beijing Solarbio Science & Technology Co., Ltd.) was used as an internal reference.

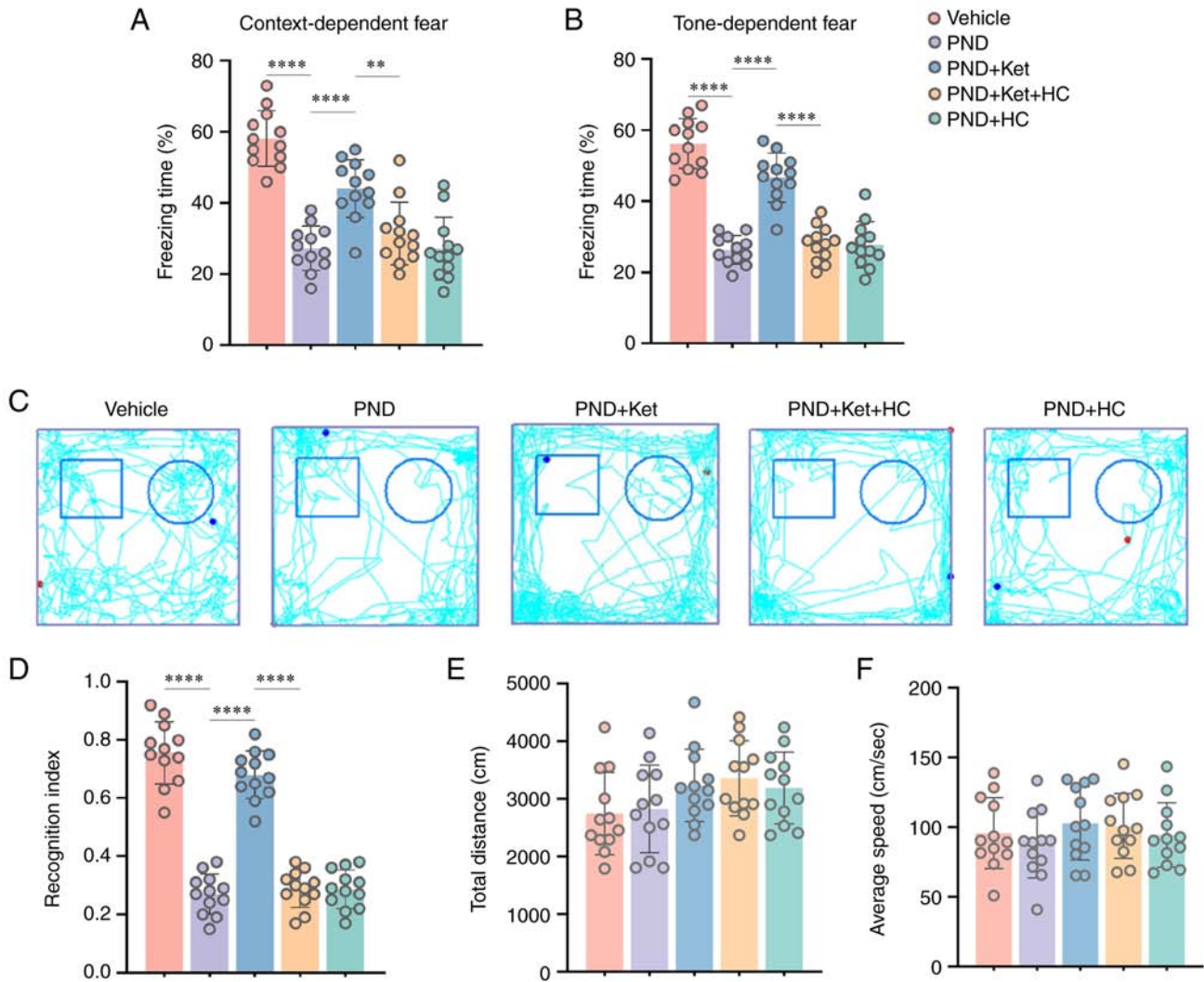


Figure 2. Ketamine administration attenuates tibial fracture surgery-induced cognitive dysfunction. The total duration of freezing behavior in the (A) context- and (B) tone-dependent fear test sessions. Data are presented as the mean  $\pm$  SD ( $n=12$ /group). (C) Trajectories generated from the novel object recognition test movement 30 days post-PND. (D) Recognition index, (E) total distance and (F) average speed in the novel object recognition test 30 days post-PND. \*\* $P<0.01$ , \*\*\*\* $P<0.0001$ . PND, perioperative neurocognitive disorder; Ket, ketamine; HC, HC-067047.

**Statistical analysis.** Unless stated otherwise, all data were expressed as mean  $\pm$  SD and analyzed using GraphPad Prism 5 (GraphPad Software, Inc.). One-way analysis of variance followed by Bonferroni's post hoc test was used to compare the differences among groups.  $P<0.05$  was considered to indicate a statistically significant difference.

## Results

**Ketamine administration alleviates tibial fracture surgery-induced cognitive dysfunction.** A total of 120 mice were involved in this current study and no mice were excluded or sacrificed prior to the endpoint. Mice exposed to tibial fracture surgery exhibited less context-related ( $P<0.0001$ ; Fig. 2A) and tone-related ( $P<0.0001$ ; Fig. 2B) freezing behaviors in terms of the total time spent motionless than vehicle-treated mice. However, these context-related (Fig. 2A) and tone-related (Fig. 2B) freezing behaviors were significantly elevated after ketamine administration ( $P<0.0001$ ); however, HC-067047, an antagonist of TRPV4 channels, could

reverse these changes ( $P=0.0027$  for context-related;  $P<0.0001$  for tone-related; Fig. 2A and B). There was no difference in the context-related and tone-related freezing behaviors in aged mice among the PND, PND + Ket + HC and PND + HC groups (Fig. 2A and B).

The novel object recognition test was performed before the fear conditioning test since the emotional effect of tone-foot shocks could influence the results of the former test (26). It is acknowledged that mice exhibit an innate tendency to explore new objects (16). Accordingly, the present study evaluated the cognition and memory of mice models of tibial fracture via the novel object recognition test. During the test phase, the mice in the PND group showed a significantly decreased RI following tibial fracture surgery compared with mice in the vehicle group ( $P<0.0001$ ; Fig. 2C and D). However, ketamine administration significantly elevated the RI in the PND + Ket group compared with in the PND group ( $P<0.0001$ ; Fig. 2C and D); this change could be reversed by HC-067047 to a certain extent ( $P<0.0001$ ; Fig. 2C and D). No difference in RI was found in aged mice among the PND, PND + Ket + HC and

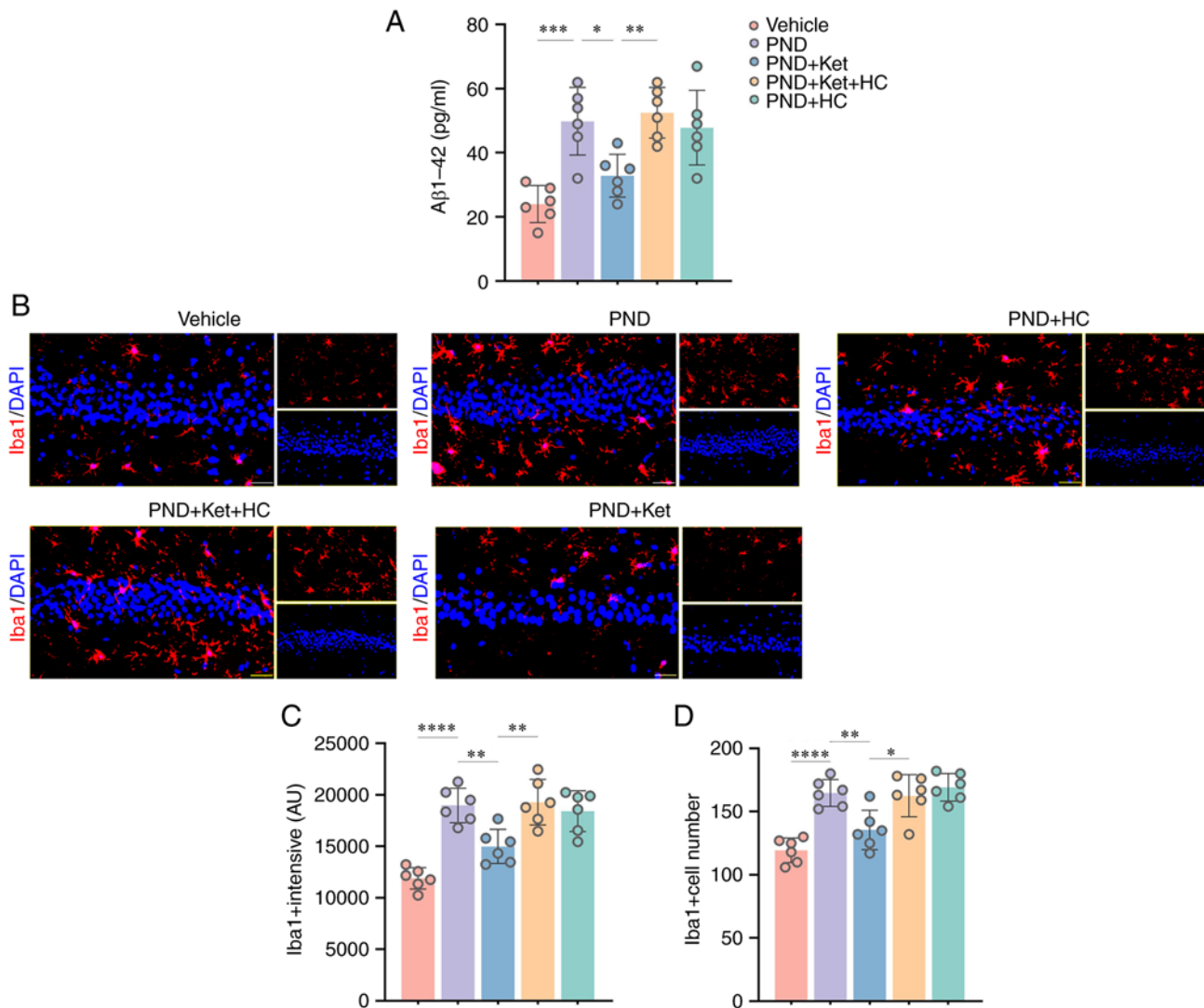


Figure 3. Ketamine administration attenuates tibial fracture surgery-induced neuronal degeneration and microglial activation. (A) Changes in A $\beta$ 1-42 in the hippocampus 30 days post-PND. (B) Representative photomicrographs of Iba1 (red) plus DAPI (blue) staining. Magnification, x400; scale bar, 50  $\mu$ m. (C) Immunofluorescence intensity and (D) number of Iba1-positive cells. Data are presented as the mean  $\pm$  SD (n=6). \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001. PND, perioperative neurocognitive disorder; Iba1, ionized calcium binding adaptor molecule 1; Ket, ketamine; HC, HC-067047.

PND + HC groups (Fig. 2C and D). In addition, no significant difference in the total distance and the average speed was found among the above five groups (Fig. 2E and F), which indicates no significant motor deficits occurred at 29 days after tibial fracture surgery.

*Ketamine administration mitigates neuronal degeneration and microglial activation after surgery.* The transmembrane glycoprotein amyloid precursor protein can be cleaved into A $\beta$  peptides by enzymes  $\beta$ -secretase and  $\gamma$ -secretase (27). Notably, A $\beta$  plaque formation has been associated with memory and cognitive dysfunction (28). In the present study, the results of an ELISA showed that A $\beta$ 1-42 levels were significantly increased in mice that underwent surgery compared with mice in the vehicle group (P=0.0003; Fig. 3A). Studies have shown that microglial activation participates in the accumulation of A $\beta$  plaque and neuronal degeneration (29). In the present study, the immunofluorescence results showed increased microglial intensity (P<0.0001; Fig. 3B and C) and number (P<0.0001; Fig. 3B and D), measured by the number of Iba1-positive

microglia in the hippocampus of mice that underwent surgery compared with the vehicle group. However, ketamine administration in the PND + Ket group significantly attenuated A $\beta$ 1-42 levels (P=0.0201; Fig. 3A) and microglial activation intensity (P=0.0050; Fig. 3B and C) and number (P=0.0055; Fig. 3B and D), compared with mice in the PND group; these changes could be reversed by HC-067047 in the PND + Ket + HC group to a certain extent (for A $\beta$ 1-42, P=0.0057; for intensity, P=0.0023; for number, P=0.0111) (Fig. 3B-D). In addition, there was no significant difference in A $\beta$ 1-42 levels, microglial intensity and number in mice among the PND, PND + Ket + HC and PND + HC groups.

*Ketamine administration mitigates oxidative stress and the inflammatory response in the early stage after surgery.* In the present study, the LPO and MDA contents were determined as biomarkers of oxidative stress, whereas the total SOD activity was assessed as an indicator of antioxidant status, as described in the literature (30,31). As shown in Fig. 4, compared with in vehicle-treated mice, a significant reduction was observed in



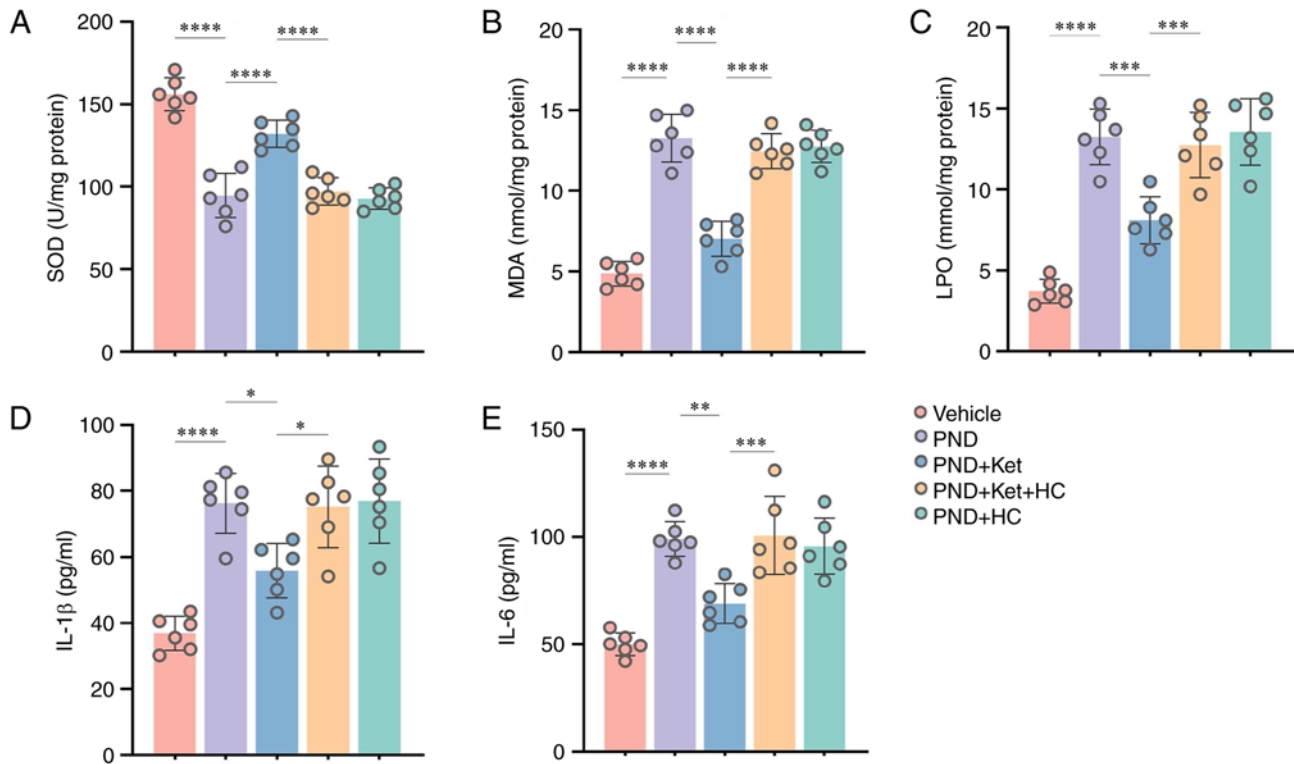


Figure 4. Ketamine administration attenuates tibial fracture surgery-induced oxidative stress and inflammatory response. Changes in the total (A) SOD, (B) MDA and (C) LPO content in the hippocampal tissue 3 days post-PND. Changes in the (D) IL-1 $\beta$  and (E) IL-6 levels in the hippocampal tissue 3 days post-PND. Data are presented as the mean  $\pm$  SD (n=6/group). \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001. SOD, superoxide dismutase; MDA, malondialdehyde; LPO, lipid peroxidation; PND, perioperative neurocognitive disorder; Ket, ketamine; HC, HC-067047.

the total SOD activity (P<0.0001; Fig. 4A), whereas the MDA (P<0.0001; Fig. 4B) and LPO contents (P<0.0001; Fig. 4C) were increased 3 days after surgery. In addition, IL-1 $\beta$  and IL-6 have been used to reflect neuronal inflammation in previous studies (32,33). IL-1 $\beta$  (P<0.0001; Fig. 4D) and IL-6 levels (P<0.0001; Fig. 4E) in the hippocampal tissue were significantly upregulated 3 days following surgery. However, compared with mice in the PND group, ketamine administration significantly restored total SOD activity (P<0.0001; Fig. 4A), and reduced the MDA (P<0.0001; Fig. 4B) and LPO contents (P=0.0001; Fig. 4C), and IL-1 $\beta$  (P=0.0118; Fig. 4D) and IL-6 levels (P=0.0013; Fig. 4E) in mice in the PND + Ket group. Notably, HC-067047 partially reversed these improvements, including total SOD (P<0.0001; Fig. 4A), MDA (P<0.0001; Fig. 4B), LPO (P=0.0005; Fig. 4C), IL-1 $\beta$  (P=0.0184; Fig. 4D) and IL-6 levels (P=0.0007; Fig. 4E) in the PND + Ket + HC group compared with in the PND + Ket group. In addition, there was no significant difference in the aforementioned indexes in mice among the PND, PND + Ket + HC and PND + HC groups.

*Protective effects of ketamine are mediated by the AMPK/NF- $\kappa$ B signaling pathway following surgery.* The protective effects induced by TRPV4 channel opening have been associated with phosphorylation of AMPK and inhibition of the NF- $\kappa$ B signaling pathway (34,35). In the present study, the effect of surgery on individual proteins in the AMPK/NF- $\kappa$ B signaling pathway was determined using western blotting with pathway-specific antibodies. It was found that tibial fracture surgery led to a significant increase in p-AMPK/total AMPK

ratio (P<0.0001; Fig. 5A and B) and p-NF- $\kappa$ B p65 levels (P<0.0001; Fig. 5A and C). Notably, ketamine administration significantly enhanced AMPK phosphorylation (P<0.0001; Fig. 5A and B) and attenuated p-NF- $\kappa$ B p65 expression in the PND + Ket group compared with in the PND group (P=0.0002; Fig. 5A and C). Conversely, HC-067047 treatment induced a significant decrease in p-AMPK/total AMPK ratio (P<0.0001; Fig. 5A and B), but a significant increase in p-NF- $\kappa$ B p65 was detected in mice in the PND + Ket + HC group compared with in the PND + Ket group (P=0.0001; Fig. 5A and C). The expression levels of the downstream inflammatory mediators of NF- $\kappa$ B, TNF- $\alpha$  and IFN- $\beta$ , were further explored by RT-Qpcr (36,37). It was shown that tibial fracture surgery led to marked elevations in TNF- $\alpha$  (P<0.0001; Fig. 5D) and IFN- $\beta$  mRNA expression (P<0.0001; Fig. 5E), whereas ketamine administration significantly attenuated TNF- $\alpha$  (P<0.0001; Fig. 5D) and IFN- $\beta$  (P<0.0001; Fig. 5E) mRNA expression levels in the PND + Ket group compared with in the PND group. By contrast, significant increases in TNF- $\alpha$  (P<0.0001; Fig. 5D) and IFN- $\beta$  (P<0.0001; Fig. 5E) mRNA expression levels were found in mice in the PND + Ket + HC group compared with in the PND + Ket group. In addition, there was no significant difference in p-AMPK, p-NF- $\kappa$ B p65, TNF- $\alpha$  and IFN- $\beta$  expression levels in mice among the PND, PND + Ket + HC and PND + HC groups (Fig. 5B-E).

## Discussion

The present study demonstrated that ketamine administration alleviated tibial fracture surgery-induced cognitive dysfunction

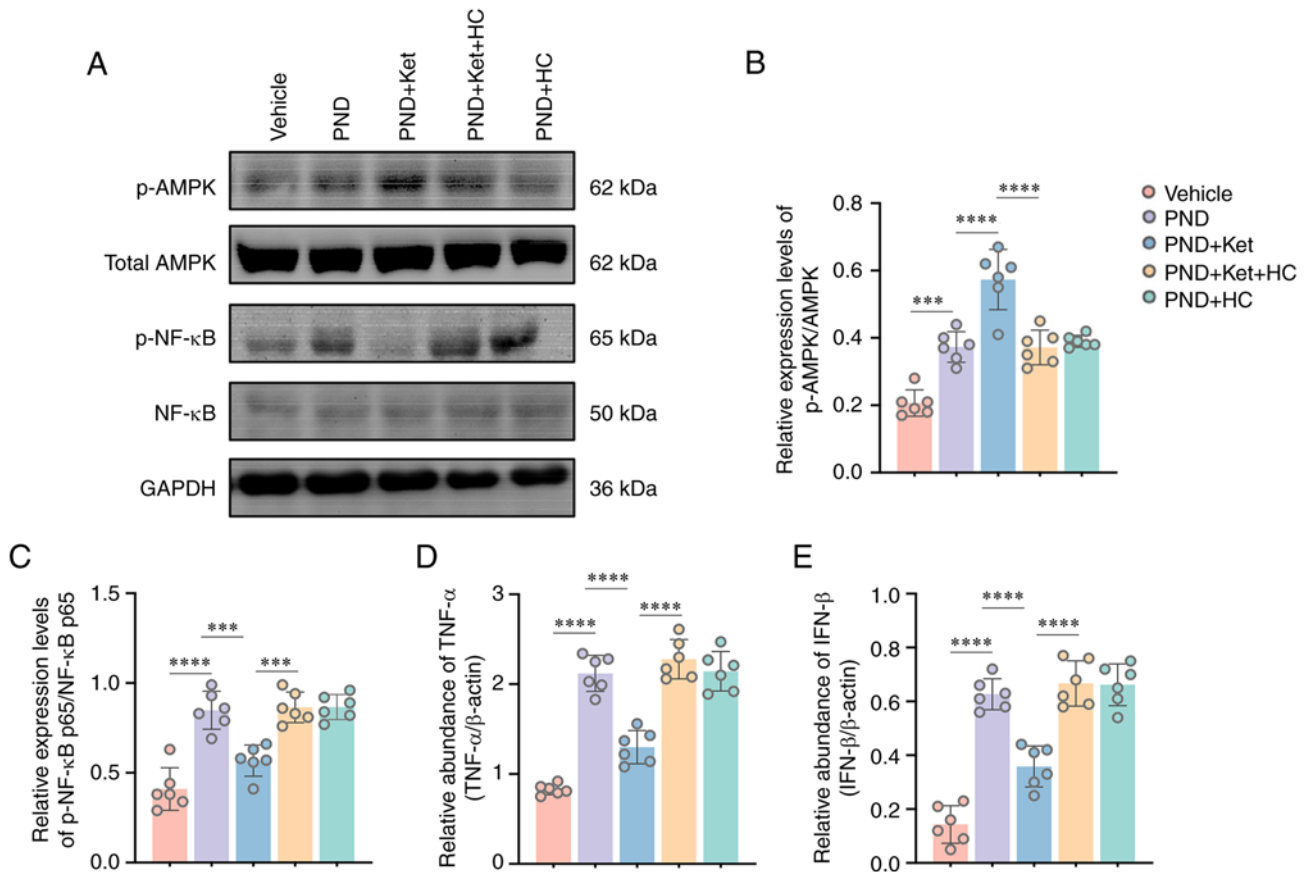


Figure 5. Ketamine regulates AMPK/NF- $\kappa$ B signaling via opening of TRPV4 channel. (A) Representative blots of p-AMPK, total AMPK, p-NF- $\kappa$ B p65 and total NF- $\kappa$ B p65 3 days post-PND. Ratio between optical density value of (B) p-AMPK/total AMPK and (C) p-NF- $\kappa$ B p65/total NF- $\kappa$ B p65 in the hippocampal tissue was evaluated by western blotting. Relative mRNA expression levels of (D) TNF- $\alpha$  and (E) IFN- $\beta$  to  $\beta$ -actin in the hippocampal tissue were evaluated by reverse transcription-quantitative PCR assay. Data are presented as the mean  $\pm$  SD (n=6/group). \*\*\*P<0.001, \*\*\*\*P<0.0001. AMPK, adenosine monophosphate-activated protein kinase; PND, perioperative neurocognitive disorder; p-, phosphorylated; Ket, ketamine; HC, HC-067047.

by triggering TRPV4 channel opening, which significantly increased the time spent exploring a novel object, and context- and tone-related freezing behaviors, whereas A $\beta$ 1-42 levels and microglial activation were attenuated. Furthermore, TRPV4 activation induced by ketamine administration decreased MDA and LPO contents, as well as IL-1 $\beta$  and IL-6 levels, whereas total SOD activity was increased. In addition, it was revealed that p-AMPK levels were upregulated, whereas p-NF- $\kappa$ B p65 protein, and TNF- $\alpha$  and IFN- $\beta$  mRNA expression levels were downregulated at an early stage following tibial fracture surgery. To a certain extent, these changes could be reversed by HC-067047, an antagonist of TRPV4 channel opening. Collectively, the present study documented the pivotal role of the TRPV4/AMPK/NF- $\kappa$ B signaling pathway in regulating oxidative stress and inflammatory response in PND (Fig. 6).

The present study explored the mechanisms of PNDs in a tibial fracture model established in aged mice. In the population of older adult humans, total joint arthroplasty of the hip and knee has become a common major surgery due to fracture or degeneration (38). It has been demonstrated that aged patients may still experience short- or long-term PND after total joint arthroplasty (39-41). A number of studies have demonstrated that tibial fracture surgery in rodents can induce the physiopathologic process of PND, subsequently inducing

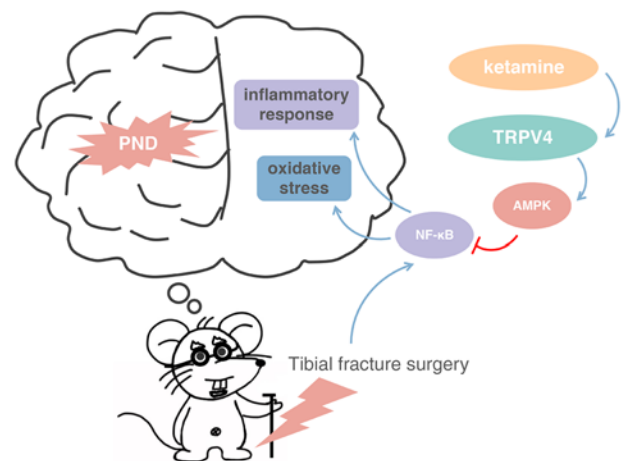


Figure 6. Schematic diagram depicting ketamine administration-induced anti-inflammation and anti-oxidative effects on PND after tibial fracture surgery, which may be mediated by the TRPV4/AMPK/NF- $\kappa$ B signaling pathway. PND, perioperative neurocognitive disorder; TRPV4, transient receptor potential vanilloid 4; AMPK, adenosine monophosphate-activated protein kinase.

significant long-term effects on cognitive dysfunction (19,42). Notably, the data of the present study revealed that tibial

fracture surgery triggered a significant decline in cognition documented by decreased context- and tone-related freezing behaviors, and attenuated the time spent exploring a novel object, which is consistent with previous studies (24,43). Once the brain is prone to disease, systemic inflammation resulting from endotoxemia, including lipopolysaccharide (LPS), can lead to an elevation in the microglial activation, subsequently contributing to neuronal death (44). There is overwhelming evidence that clinically relevant tibial fracture surgery in rodent models can cause memory dysfunction through induction of systemic cytokine release and disruption of the blood-brain barrier (BBB), which allows macrophages to migrate into the central nervous system and shifts microglial morphology (45,46). In addition, systemic inflammation induced by tibial fracture surgery has been suggested to be a vital initiator of neuroinflammation and delirium-like behavior (47). Notably, several studies have suggested that PND following surgery may be associated with neuronal degeneration and microglial activation (48,49). In the present study, tibial fracture surgery significantly increased A $\beta$ 1-42 levels and exacerbated microglial activation in the hippocampus. These results suggested that cognitive dysfunction was successfully triggered in the PND model, and neuronal degeneration and microglial activation in the hippocampus were related to PND.

Oxidative stress and inflammatory response have been demonstrated to participate in the pathophysiology of PNDs. Several studies have reported that following surgery, oxidative stress and inflammatory response can lead to neuronal degeneration and microglial activation (16,19,50). Furthermore, it has been reported that proinflammatory cytokines, such as IL-1 $\beta$  and IL-6, can lead to neuronal damage during ischemia or infection (51-53). Proteins, lipids and nucleic acids can be modified by free radicals and lipid peroxidation following activation of the central nervous system and the immune system under inflammatory conditions (19). Given the high metabolic rate and peroxidation induced by fatty acids and the low antioxidant capacity, neurons are more susceptible to the destructive effects of oxidative stress (54). In this regard, BBB dysfunction following surgery reportedly facilitates entry of inflammatory factors and oxidative species in the central nervous system, resulting in microglial activation (55). In addition, lipid peroxidation products, along with proinflammatory factors released from activated microglia, form a toxic environment for neurons (56). Furthermore, the present study demonstrated that oxidative stress (LPO and MDA) and inflammatory factors (IL-1 $\beta$  and IL-6) were significantly increased at an early stage following tibial fracture surgery. Recently, it was reported that the occurrence of an inflammatory response in neurons contributes to neuronal degeneration and cognitive dysfunction following surgical exposure (57). Besides neurons, glial cells, including astrocytes and microglia, generally exhibit a quiescent phenotype under normal physiological conditions and can be activated after peripheral surgery and secrete appreciable levels of pro-inflammatory cytokines within the brain (58). An increasing body of evidence has suggested that postoperative astrocytic and microglial activation participate in oxidative stress at the early stage of PND (47,59,60). In addition, mast cells, vital sentinel cells for host defense

against selected pathogens, are typically found in the choroid plexus, meninges and the brain side of the BBB (61). Notably, there is a definite communication between neurons, glia and mast cells in the process of PND (62). It is suggested that a marked attenuation in BBB permeability can be induced through pharmacological inhibition of mast cells, which contributes to neuroinflammation under PND (63). Although which cell types in the brain exhibit changes in oxidative stress and inflammatory response should be further explored, these findings suggested that oxidative stress and inflammatory response may participate in cognitive dysfunction after tibial fracture surgery.

Ketamine has been reported to act as a neuroprotective agent that can inhibit the inflammatory response, oxidative stress and cellular dysfunction (64-66). It was suggested that ketamine could attenuate LPS-induced injury in BV2 cells via inhibiting NMDA receptors, and reducing Ca<sup>2+</sup> levels and NF- $\kappa$ B phosphorylation (67). Under hypoxic conditions, ketamine can attenuate inflammatory pathways, mirrored by decreased IL-6 and IL-1 $\beta$  levels (68). In addition, ketamine may suppress oxidative stress via activation of Nrf2 and its downstream proteins in a rodent model of traumatic brain injury, thus exhibiting neuroprotective effects (64). The results of the present study indicated that both AMPK phosphorylation and NF- $\kappa$ B phosphorylation were increased after surgery. The mechanism may be associated with NF- $\kappa$ B phosphorylation can lead to AMPK phosphorylation (69). It has been suggested that ketamine can exert a rapid antidepressive effect via the phosphorylation of AMPK and its downstream proteins, including brain-derived neurotrophic factor (70). In addition, studies have shown that AMPK phosphorylation exerts an excellent analgesic effect by inhibiting the NF- $\kappa$ B signaling pathway (71) and ketamine administration suppresses endotoxin-induced NF- $\kappa$ B phosphorylation both *in vivo* and *in vitro* (72). The results of the present study indicated that ketamine administration significantly increased AMPK phosphorylation, but decreased p-NF- $\kappa$ B p65, as well as the expression levels of the downstream mediators TNF- $\alpha$  and IFN- $\beta$ . These results revealed that the ketamine-induced improvement in cognitive dysfunction may be mediated by suppressing the AMPK/NF- $\kappa$ B signaling pathway.

TRPV4 channels have been reported to be chemical, mechanical stimuli and osmotic sensors, and changes in the internal environment can induce TRPV4 activation (73,74). TRPV4 can sense mechanical stimulation, eicosanoid metabolites and cell swelling, and then can adjust its opening state (75). Studies have demonstrated that internal environment changes, such as oxidative stress and inflammation, can activate TRPV4 channels opening (12,76,77). The present study showed that treatment with an antagonist of TRPV4 channels reversed the inhibition of oxidative stress and inflammatory response in mice administered with ketamine after tibial fracture surgery. In addition, the antagonist significantly attenuated ketamine-induced inhibition of AMPK/NF- $\kappa$ B signaling following tibial fracture surgery. Moreover, the antagonist of TRPV4 channel opening abolished the improvement in cognitive dysfunction. The current data only showed the pivotal role of the TRPV4/AMPK/NF- $\kappa$ B signaling pathway in regulating oxidative stress and inflammatory response in PND through HC-067047, an antagonist



of TRPV4. However, there is a limitation in that inhibitors specific to AMPK pathways, such as compound C and MRT199665, should be further investigated to determine underlying AMPK signal mechanisms of ketamine (78,79). The present study indicated inhibition of TRPV4 channel opening may be associated with the neuroprotective effects of ketamine under PND conditions.

In conclusion, ketamine administration improved cognitive dysfunction in mice that underwent tibial fracture surgery, and the mechanism may involve inhibition of oxidative stress and inflammatory response mediated by suppression of the TRPV4/AMPK/NF- $\kappa$ B signaling pathway. Notwithstanding that the present study provided compelling evidence that ketamine has huge prospects for clinical application in PND treatment, further studies are required to explore the underlying mechanisms.

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### Availability of data and materials

All data generated and analyzed during this study are included in this published article.

### Authors' contributions

QL was responsible for methodology, funding acquisition, data analysis and writing the original draft. YQT was responsible for methodology, formal analysis and software. XWW was responsible for design of methodology and formal analysis. DQP was responsible for design of methodology and analysis of immunofluorescence staining. YX was responsible for methodology and formal analysis. DNZ was responsible for conceptualization, supervision, writing, review and editing. QL and DNZ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

The present study was carried out in compliance with the ARRIVE guidelines. Experiments were performed according to institutional and national guidelines, and were approved by the Animal Ethics Committee of The Second Affiliated Hospital of Jiaying University.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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