Capsaicin attenuates sepsis-associated encephalopathy by inhibiting neuroinflammation and apoptosis whilst activating mitophagy through the BNIP3/NIX pathway

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Abstract. Sepsis-induced abnormalities in brain function or sepsis-associated encephalopathy (SAE) can manifest as cognitive dysfunction and other neuropsychiatric symptoms; however, the underlying mechanisms remain unclear. The aim of the present study was to elucidate the possible effects and mechanism of capsaicin, a transient receptor potential vanilloid 1 (TRPV1) agonist, on the pathological features of SAE. A model of SAE in C57BL/6 mice was generated using cecal ligation and puncture (CLP). Capsaicin (1 mg/kg) was injected subcutaneously before surgery. Cognitive function in mice was evaluated using the novel object recognition test (NORT) and Morris water maze (MWM). Immunofluorescence staining, ELISA, western blotting and transmission electron microscopy were performed to detect the degree of microglial activation (ionized calcium-binding adapter molecule 1), proinflammatory cytokine levels (TNF-α), autophagy and apoptosis-related protein expression, and autophagosomes. Autophagic flux was monitored using the LC3-GFP-mCherry fluorescent reporter. Compared with that in the sham group mice, the expression levels of TRPV1 were significantly reduced in the hippocampal tissue of mice with sepsis. Mice with sepsis also exhibited cognitive dysfunction. Notably, a single administration of capsaicin reduced the mortality rate, but did not improve cognitive function in mice with sepsis. Furthermore, repeated administration of capsaicin was revealed to enhance the recognition index of novel objects among mice with sepsis, to reduce the latency to locate the platform and to augment the duration of mouse platform quadrant movements, according to the NORT and MWM tasks. Increased microglial activation, release of proinflammatory cytokines and expression levels of apoptosis-related proteins were all observed in mice with CLP-induced sepsis, as was brain tissue destruction in the hippocampal regions. By contrast, capsaicin treatment ameliorated CLP-induced microglial activation, inflammation, neuronal apoptosis (cleaved caspase 3 expression increased) and brain tissue destruction. Furthermore, application of capsaicin increased the expression levels of LC3, reduced the expression of p62 and elevated autophagic flux compared with those in the CLP group. Finally, treatment with capsaicin effectively enhanced the levels of Bcl-2-interacting protein 3 (BNIP3) and BNIP3-like (NIX) expression. These findings suggested that capsaicin may be considered a potential drug for the treatment of SAE, and BNIP3/NIX-mediated mitophagy may be involved in this process.

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Introduction

Sepsis is a life-threatening condition associated with multi-organ dysfunction caused by the dysregulated host response to infection (1). According to 2017 statistics, there were 48.9 million cases of sepsis and 11 million cases of sepsis-related mortality worldwide, and sepsis accounted for ~20% of all global deaths (2). Sepsis-induced multi-organ dysfunction refers not only to peripheral organ damage, but also includes central nervous system complications, which are associated with the common symptom of cognitive dysfunction, which negatively affects patient survival (3). Sepsis-associated encephalopathy (SAE) is a type of acute brain dysfunction caused by sepsis, which frequently causes confusion to progress to delirium and even coma (4). In total, 70% of patients with sepsis can develop SAE. The development of encephalopathy increases the likelihood of mortality in patients with sepsis (5), the mechanisms underlying the association between the two remain to be fully elucidated. Notably, it is considered that the more severe the encephalopathy, the

higher the mortality rate (6). In addition, 10% of sepsis survivors continue to suffer from chronic cognitive dysfunction within 3 years after hospital discharge (7), and SAE increases the risk of suicide within 2 years after recovery (8). Survivors of sepsis often exhibit long-term cognitive deficits and psychological disorders that seriously affect their quality of life and morbidity (9). Therefore, the early recognition and diagnosis of SAE, in addition to its effective treatment, have important research value and social significance.

Currently, numerous pathogenic mechanisms of SAE have been documented, including blood-brain barrier (BBB) damage, neurotransmitter disorders, oxidative stress, neuroinflammation and neuronal apoptosis, all of which interact in a complex manner to aggravate cognitive impairment (10). However, due to its highly complex pathophysiological characteristics, specific treatment regimens remain elusive. Therefore, an in-depth study of the pathophysiological mechanisms of SAE, and an exploration of novel methods to effectively prevent and treat SAE have become research hotspots in this field. Microglia-mediated neuroinflammation is a key factor in the pathogenesis of SAE (11). Subsequently, inflammatory signals, such as TNF-α and IL-1β, enter the brain in both neurological and humoral manners following sepsis, resulting in the activation of glial cells and the release of substantial quantities of inflammatory factors such as IL-6, TNF-α and IL-1β. This in turn leads to the further exacerbation of inflammation, disruption of BBB integrity and neuronal cell death (12). Consequently, modulation of microglia activity has emerged as a promising therapeutic approach for the management of SAE.

Mitophagy is a form of selective autophagy that removes aged or damaged mitochondria in response to a stimulus. Mitophagy induces the clearance of damaged mitochondria through an autophagic mechanism, thereby contributing to mitochondrial quality control, and the maintenance of cellular and mitochondrial homeostasis in inflammatory diseases (13,14). Mitochondrial autophagy mainly consists of PTEN-induced kinase 1 (PINK1)/Parkin-dependent and PINK1/Parkin-independent [such as Bcl-2-interacting protein 3 (BNIP3)/BNIP3-like (NIX) and FUN14 domain-containing protein 1] autophagic pathways (15). In sepsis, mitochondria are particularly susceptible to damage, after which the inner mitochondrial membrane potential is reduced, damaged mitochondria are enclosed into autophagosomes and ultimately degraded by fusion with lysosomes, thereby facilitating the recovery of sepsis-affected organ function (16,17). During the early stages of sepsis, mitophagy is enhanced, such that mitochondrial danger-associated molecular patterns and reactive oxygen species production is reduced, and mitochondrial mass is controlled, thereby ameliorating sepsis-induced abnormalities (18). Mitophagy may therefore be a novel potential target for sepsis therapy.

Transient receptor potential vanilloid 1 (TRPV1) is a member of the non-selective cation channel family that can be activated by pH, noxious heat, capsaicin and vanilloid compounds (19). TRPV1 expression is widely distributed in the peripheral and central nervous systems (20). Capsaicin has been reported to exert neuroprotective effects. It has been shown to reduce oxidative stress and behavior impairment, reduce the aggregation of misfolded proteins and improve mitochondrial function (21,22). Capsaicin, which is the principal active ingredient in chili peppers, has garnered attention due to its potential for the treatment of sepsis. Capsaicin has been observed to protect against sepsis-induced liver, lung, kidney and cardiac injury by attenuating mitochondrial dysfunction, apoptosis and pyroptosis, inhibiting the NF-κB pathway, suppressing oxidative stress and inflammation, and modulating autophagy (23-26). Capsaicin has also been demonstrated to directly inhibit the pyruvate kinase isozymes M1/M2/lactate dehydrogenase A-mediated Warburg effect and to reduce glycolytic metabolism in inflammatory macrophages, thereby attenuating the excessive inflammatory response in a septic setting (27). In the central nervous system, targeting TRPV1-mediated autophagy may alleviate multiple disease-related symptoms, such as Alzheimer's disease, ischemia-reperfusion injury and Parkinson's disease (28-30). *In vitro*, inhibition of TRPV1 has been shown to promote mitophagy and regulate mitochondrial function in multiple myeloma cell lines (31). By contrast, capsaicin has been observed to induce mitochondrial loss and activate mitophagy in PC12 cells in a dose-dependent manner (32), suggesting that TRPV1-mediated mitophagy can affect mitochondrial structure and function. However, *in vivo*, the role of TRPV1-mediated mitophagy and whether it is involved in SAE remains unknown. Consequently, the present study aimed to investigate the potential role of TRPV1-mediated mitophagy in SAE.

Materials and methods

Animals. Male C57BL/6 mice (age, 8-10 weeks; weight, 20-25 g) were obtained from GemPharmatech Co., Ltd. All mice were housed in a cage under a 12-h dark/light cycle at a room temperature of 23±1˚C and 55% humidity; and all mice had free access to food and water. Following the entrustment of the management and operation of the animal facility to China Technology Industry Holdings (Shenzhen) Co., Ltd., all experiments were approved by the Committee on the Animal Research Ethics of China Technology Industry Holdings (Shenzhen) Co., Ltd. (Shenzhen, China; approval no. 202200105) The present study was conducted in accordance with the ARRIVE guidelines (33). A total of 148 C57BL/6 male mice were used in the present study and were randomly divided into the following three experimental groups: Sham group (no ligation and puncture; n=38), cecal ligation and puncture (CLP) group (n=55) and CLP combined with capsaicin group (n=55). Capsaicin (cat. no. HY-10448; MedChemExpress) was dissolved in a solution containing 10% DMSO (v/v), 40% PEG300 (v/v), 5% Tween-80 (v/v) and 45% saline (v/v). A subcutaneous (s.c.) injection of capsaicin at 1 mg/kg was administered once 1 h before CLP surgery or three times at different time points (1 h, 1 day and 2 days before CLP surgery). Mice in the sham group were injected with an equal amount of saline vehicle. The total duration of the animal study was 19 days. Mice were then sacrificed after the Morris water maze (MWM) experiments, and hippocampal tissue was removed for western blotting and immunofluorescence analyses. The health of the mice was monitored daily throughout the experiment, and mice were euthanized if they reached any of the following humane endpoints: Weight loss

>20%, persistent refusal to eat or drink for >24 h, and signs of respiratory distress. The mice were confirmed dead by an absence of pain responses, complete cessation of cardiac and respiratory activity, and pupil dilation.

CLP model. Mice were subjected to CLP according to a previously described method (34). Briefly, following anesthesia of the mice with isoflurane (2% induction, 1.5% maintenance) fur was removed from the abdomen and it was sterilized with 75% alcohol. An incision of ~1.5 cm was then made along the midline of the abdomen, where the cecum was isolated and removed using blunt dissecting forceps, leaving the remaining small and large intestines in the abdominal cavity. The procedure was conducted with the utmost care to avoid any disruption or damage to the mesenteric vessels. Subsequently, the cecum was ligated at a point 50% of its length from the tip, where a 21G needle was used to puncture the cecum at a point midway between the ligation and the tip. The puncturing of blood vessels was avoided. Following the removal of the needle, a minimal quantity of feces was expressed to confirm patency. It was imperative that the cecum be repositioned within the abdominal cavity in such a way that the feces did not spread from the cecum to the edge of the abdominal wall. The abdominal cavity was then closed layer by layer using sutures. To compensate for the loss of body fluids, saline (5 ml/100 g body weight; s.c.) was injected. Additionally, buprenorphine HCl (0.05 mg/kg body weight; s.c.) was administered for postoperative analgesia after completion of the surgery. Thereafter, the animal was placed back into the cage. If a mouse exhibited prolonged lethargy and/or did not eat or drink for 24 consecutive hours, they were evaluated and if it was determined that they would die of sepsis, they were euthanized. Mice were euthanized by deep anesthesia with sodium pentobarbital [120 mg/kg, intraperitoneal (i.p.)] followed by cervical dislocation. The researchers made every effort to minimize the number and suffering of the animals for all experiments. Survival rates were 100% in the sham group and ~53% in the sepsis group, which is consistent with previous studies (16,35).

Novel object recognition test (NORT). NORT is a tool used to assess the learning and memory abilities of experimental animals (16). To evaluate the exploration drive of mice towards novel objects, the animals were placed in boxes (50x50x50 cm) within an open field experimental setup. The boxes were made of white polyethylene walls and a white polyethylene floor. Before and after each trial, the boxes and objects were cleaned with ethanol to remove any residual odors or substances; it was important to ensure that no traces of feces or odor remained from the previous mouse. Over the course of 2 consecutive days, the mice were allowed to acclimate to the empty boxes for 10 min each day. Subsequently, the mice were placed in boxes containing two different objects (A and B) for 5 min each. The number of times each object was explored was recorded, representing the sample phase. This process was repeated every 2 h. During this period, the mice were returned to their cages while the objects that had been explored the fewest times were replaced with a different object (C). If a mouse stayed in the corner and did not explore objects, it was removed, 3 mice in CLP group and 4 mice in CLP + capsaicin group were removed. After 2 h, the mice were released into the boxes and allowed to explore freely for 5 min, before the number of times each object was explored was recorded, representing the acquisition phase. The discrimination index was calculated as the number of times a novel object was explored divided by the total number of explorations.

MWM. This task was performed according to a previously described method (36). The MWM consisted of a circular pool (diameter, 100 cm; height, 38 cm) and a removable circular platform (diameter, 6 cm); the pool was divided into four quadrants with the circular platform fixed in the third quadrant. Each quadrant had a clear pattern on the wall of the pool and the temperature of the pool was controlled at 21±1˚C. The entire process included a training and testing phase, the training phase was further divided into the platform visibility and platform hiding period.

The initial stage was the platform visibility period, which lasted for 1 day. During this stage, the platform was situated at a height of 1 cm above the water surface. The mice were trained four times per day, with each training session lasting for 1 min. The mice were gently guided into the water from the midpoint of the four quadrants facing the pool wall and allowed to explore freely for 1 min. The mice were permitted to locate the platform and remain there for 15 sec. In the event that the mice were unable to find the platform within 1 min, they were assisted in locating it and were allowed to remain on it for 15 sec. At the conclusion of the training period, the mice were dried with paper towels and transferred to the pre-prepared warm and dry cages.

The second phase was the platform-hiding period, which lasted for 4 days. During this period, the water surface was maintained at a height of 1 cm above the platform, with the remaining steps consistent with those employed during the platform visibility period.

The third stage was the test phase, which lasted for 1 day. During this stage, the platform was removed and the mice were placed at the midpoint of the edge of the opposite quadrant of the pool and allowed to explore freely for 1 min. The number of times the mice crossed the platform and the residence time in the quadrant where the platform was located were analyzed using the software (EthoVision XT 17.5; Noldus Information Technology BV).

Cell culture and treatment. BV2 cells were obtained from Shenzhen University (Shenzhen, China) and were incubated in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 2% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin, (100 U/ml; Gibco; Thermo Fisher Scientific, Inc.). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich; Merck KGaA. Capsaicin was dissolved in DMSO. LPS was dissolved in PBS. BV2 cells were categorized into three groups: Ctrl, LPS and capsaicin + LPS. The cells were cultured in the presence or absence of LPS (1 μg/ml) for 12 h at 37˚C, then further treated with capsaicin (10 μM) for 24 h at 37˚C.

Immunofluorescence staining. For brain section staining, three groups (Sham, CLP + Veh and CLP + capsaicin) of mice (6 mice per group) were anesthetized with sodium pentobarbital (65 mg/kg; i.p.), and perfused transcardially with cold 4% paraformaldehyde. Next, the whole brain was extracted,

and post-fixed with 4% formaldehyde overnight at 4˚C, before being dehydrated with 30% sucrose solution. The brain tissues were sectioned coronally at 30 μm with a cryostat (Leica Microsystems GmbH). Subsequently, the sections were permeabilized with 0.1% Triton X-100 in PBS and blocked with freshly prepared blocking solution [3% donkey serum (cat. no. SL050; Beijing Solarbio Science & Technology Co., Ltd.) and 0.2% Triton X-100 in PBS] for 1 h at room temperature, followed by incubation with the primary antibody, anti-ionized calcium-binding adapter molecule 1 [Iba1; cat. no. ab5076; research resource identifier (RRID), AB_2224402; 1:500; Abcam], overnight at 4˚C. After washing with PBS, secondary antibody conjugated with Alexa Flour 488 (cat. no. A-11055; 1:500; Thermo Fisher Scientific, Inc.) was added for incubation for 2 h at room temperature. Slices were counterstained with DAPI (cat. no. C1005; Beyotime Institute of Biotechnology) for 5 min at room temperature and mounted with Vectashield Antifade mounting medium (cat. no. H1000-10; Vector Laboratories, Inc.). Immunofluorescence images were captured by confocal microscopy (LSM 800; Carl Zeiss AG) and the experiments were repeated three times. The numbers and area of soma on microglia were quantified using ImageJ 1.54g software (National Institutes of Health).

Western blotting. Hippocampal tissues (~30 mg) were homogenized in RIPA buffer (Beyotime Institute of Biotechnology) containing protease and phosphatase inhibitors (Roche Diagnostics GmbH) on ice and stored at -80˚C until use. The protein concentrations were quantified using a BCA kit (cat. no. 23225; Thermo Fisher Scientific, Inc.) (16). Subsequently, 30 μg protein was separated on 7.5, 10 and 15% gels by SDS-PAGE and transferred onto PVDF membranes (Merck KGaA). The membranes were blocked with 5% non-fat milk for 1 h at room temperature, before being incubated with primary antibodies overnight at 4˚C. After washing with TBS-0.1% Tween-20, the membranes were incubated with the corresponding HRP-conjugated secondary antibodies (anti-rabbit; cat. no. 7074S; 1:1,000; anti-mouse; cat. no. 7076S; 1:1,000; Cell Signaling Technology, Inc.) at room temperature for 1 h. The bands were detected with a chemiluminescent reagent (MilliporeSigma). The experiments were repeated three times. The following primary antibodies were used: TRPV1 (cat. no. NB100-1617; RRID: AB_10002124; 1:1,000; Bio-Techne), LC3B (cat. no. T55992; RRID: AB_2929010; 1:500; Abmart Pharmaceutical Technology Co., Ltd.), p62 (cat. no. 23214; RRID: AB_2798858; 1:500; Cell Signaling Technology, Inc.), Parkin (cat. no. A11172; RRID: AB_2758446; 1:500; ABclonal Biotech Co., Ltd.), PINK1 (cat. no. A7131; RRID: AB_2767686; 1:500; ABclonal Biotech Co., Ltd.), pro-caspase 3 (cat. no. ET1608-64; RRID: AB_3069820; 1:500; HUABIO), cleaved caspase 3 (cat. no. BF0711; RRID: AB_2846190; 1:500; Affinity Biosciences), nucleotide-binding oligomerization domain, leucine rich repeat and CARD domain-containing 4 (NLRC4; cat. no. A7382; RRID: AB_2767914; 1:500; ABclonal Biotech Co., Ltd.), BNIP3 (cat. no. 68091-1-Ig; RRID: AB_2918828; 1:2,000; Proteintech Group, Inc.), NIX (cat. no. 12986-1-AP; RRID: AB_2877901; 1:1,000; Proteintech Group, Inc.), β-actin (cat. no. 4970S; RRID: AB_2223172; 1:3,000, Cell Signaling Technology, Inc.) and β-tubulin (cat. no. 2148S; RRID: AB_823664; 1:3,000, Cell Signaling Technology, Inc.).

ELISA. The hippocampal tissues were collected and stored at -80˚C until needed. The levels of TNF-α were measured using an ELISA kit (cat. no. E-EL-M3063; Elabscience Bionovation Inc.) according to the manufacturer's instructions.

Hematoxylin and eosin (H&E) staining. A total of six mice per group were anesthetized with sodium pentobarbital (65 mg/kg; i.p.), and perfused transcardially with 4% paraformaldehyde in PBS resulting in mortality. Brain tissue was extracted and post-fixed with 4% paraformaldehyde for 24 h at 4˚C and embedded in paraffin. The brain tissues were then sliced into 8-μm sections. The Hematoxylin-Eosin Stain kit (cat. no. G1076; Wuhan Servicebio Technology Co., Ltd.) was used for H&E staining according to the manufacturer's protocol. All images were captured using an ECLIPSE E100 microscope (Nikon Corporation). Hippocampal neuronal damage was evaluated by a researcher blinded to each group, based on the following observations (37): Grade 0, no damage to any hippocampal subregion; grade 1, scattered neurons were damaged in the CA1 subregion; grade 2, moderate numbers of damaged neurons in the CA1 subregion; grade 3, severe damage to pyramidal cells in the CA1 subregion; and grade 4, extensive cell damage in all hippocampal regions.

Transmission electron microscopy (TEM). Hippocampal tissues were fixed with 2.5% glutaraldehyde for 24 h at 4˚C (16). After washing with 0.1 M PBS (pH 7.4) three times for 15 min each, the tissues were post-fixed with 1% OsO4 in 0.1 M PBS (pH 7.4) for 2 h at room temperature, followed by dehydration with ethanol and embedding in Acetone-EMBed 812 resin for penetration for 2-4 h at 37˚C. After polymerization, the semi-thin slices were used for positioning, and then cut into 60-80-nm ultra-thin sections. The sections were then stained with 2% uranium acetate and 2.6% lead citrate for 8 min each at room temperature before being observed under TEM (HT7800; Hitachi, Ltd).

Autophagic flux measurements. BV2 cells were plated into 24-well plates at a density of $1x10^5$ cells/well and were cultured overnight. Subsequently, the cells were infected with LC3-GFP-mCherry lentivirus (MOI, 40; OBiO Technology (Shanghai) Corp., Ltd.) for 72 h at 37˚C (38). Following a change of medium, the virus-transfected cells were randomly divided into the Ctrl, LPS and LPS + capsaicin groups. LPS (1 μg/ml) was added to the LPS and LPS + capsaicin groups for 12 h. The Ctrl groups were given the same volume of saline. The cells were incubated with 10 μM capsaicin for 24 h and were then fixed with 4% paraformaldehyde for 30 min at room temperature and stained with DAPI (1 μg/ml) for 15 min at room temperature. Immunofluorescence images were captured using a confocal microscope (LSM 800; Carl Zeiss AG), with yellow fluorescence representing autophagosomes and red fluorescence representing autophagolysosomes. The experiments were repeated three times.

Statistical analysis. With the exception of the neuronal damage scores, all data are presented as the mean ± standard deviation and analyzed using GraphPad Prism 9.0 software (Dotmatics). Neuronal damage scores are presented as median values with interquartile ranges. Statistical significance was estimated

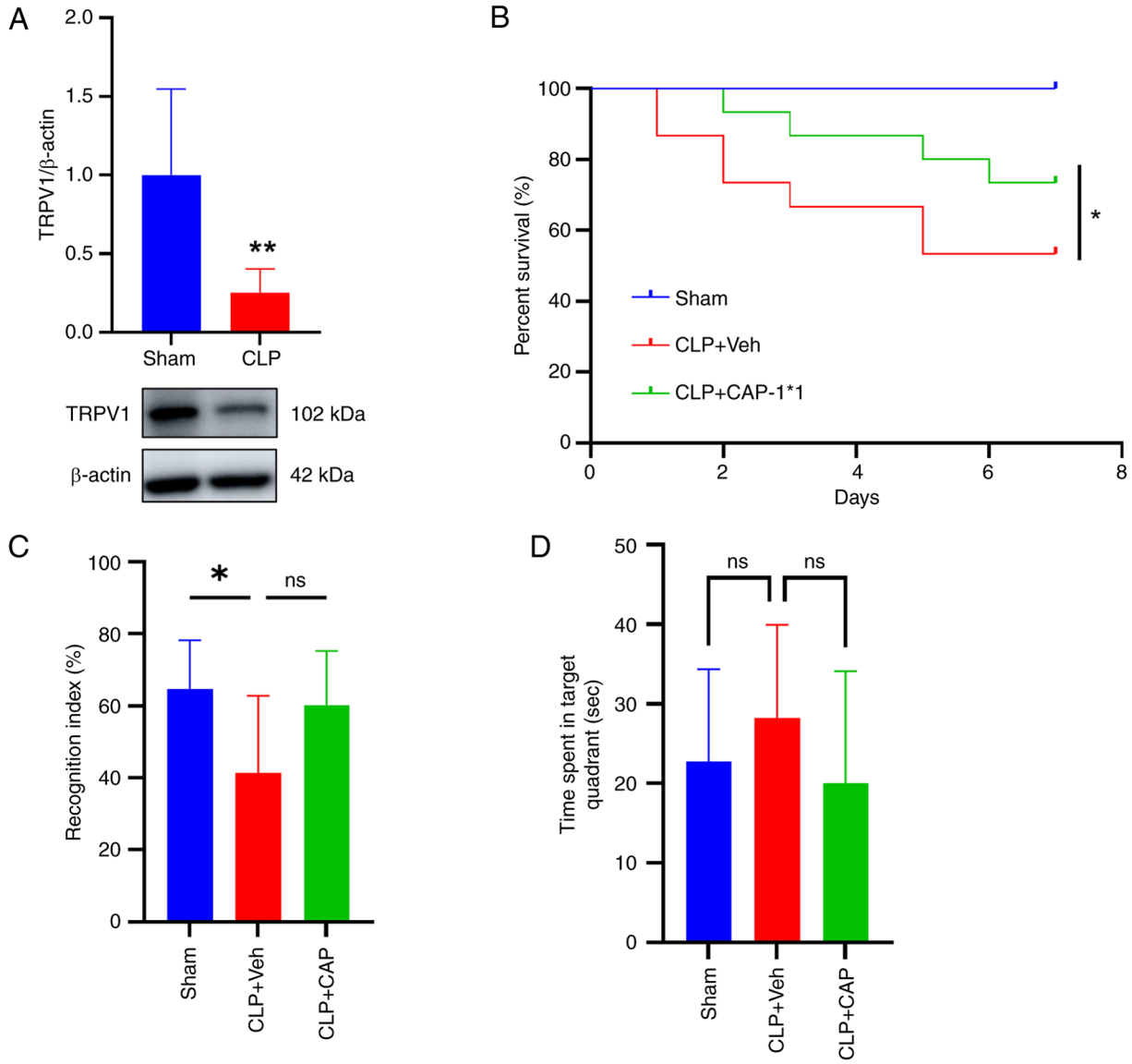

Figure 1. Effects of subcutaneous CAP administration on mouse survival rate and cognitive function after CLP surgery. (A) Western blot analysis and semi-quantitative analysis of TRPV1 protein expression levels in hippocampal tissues after CLP surgery (n=6 mice/group; unpaired Student's t-test; $t_{(10)}$=3.221; P<0.01). (B) Survival rate of sham (n=10), CLP + Veh (n=15) and CLP + 1 mg/kg CAP (n=15) mice were monitored for 7 days. Log-rank Mantel-Cox test, P=0.035. (C) Recognition index of the novel objective in the novel object recognition test [Sham (n=8), CLP (n=6) and CLP + CAP (n=7) mice; one-way ANOVA with Bonferroni post hoc test; $F_{(2,18)}$=3.670; P=0.046]. (D) Time spent in the target quadrant in the Morris water maze test phase [Sham (n=8), CLP (n=10) and CLP + CAP (n=12) mice]. Data are presented as the mean ± standard deviation. *P<0.05 and **P<0.01 vs. sham. CAP, capsaicin; CLP, cecal ligation and puncture; TRPV1, transient receptor potential vanilloid 1; Veh, vehicle; CAP-1*1, capsaicin 1 mg/kg * 1 application.

using unpaired Student's t-test or one-way ANOVA followed by Bonferroni post hoc test. To assess neuronal damage score, Kruskal-Wallis test followed by Dunn's multiple comparisons test was used. The Kaplan-Meier method was used for survival analysis, and differences were analyzed using the log-rank (Mantel-Cox) test. P<0.05 was considered to indicate a statistically significant difference.

Results

Capsaicin ameliorates cognitive deficits in mice with sepsis. Initially, TRPV1 expression was measured in the hippocampus of mice with CLP-induced sepsis, and the expression levels of TRPV1 were significantly reduced in mice in the CLP group compared with those in the sham group (Fig. 1A), suggesting that TRPV1 expression in the hippocampus may be associated with CLP-induced SAE. Next, the possible effect of capsaicin, a TRPV1 agonist, on the learning and memory of mice with sepsis was assessed. Specifically, NORT and MWM test were conducted to assess cognitive function. The s.c. administration of capsaicin (1 mg/kg) 1 h before surgery significantly improved the survival rate of mice (Fig. 1B). According to NORT, the recognition index was significantly reduced after CLP surgery compared with that in the sham group; however, a single application of capsaicin did not ameliorate the recognition index compared with that in mice from the CLP group (Fig. 1C). Consistent with the NORT results, MWM experiments demonstrated that there was no significant difference in the time spent in the platform quadrant between the CLP group and the sham group. Moreover, mice in the capsaicin-treated

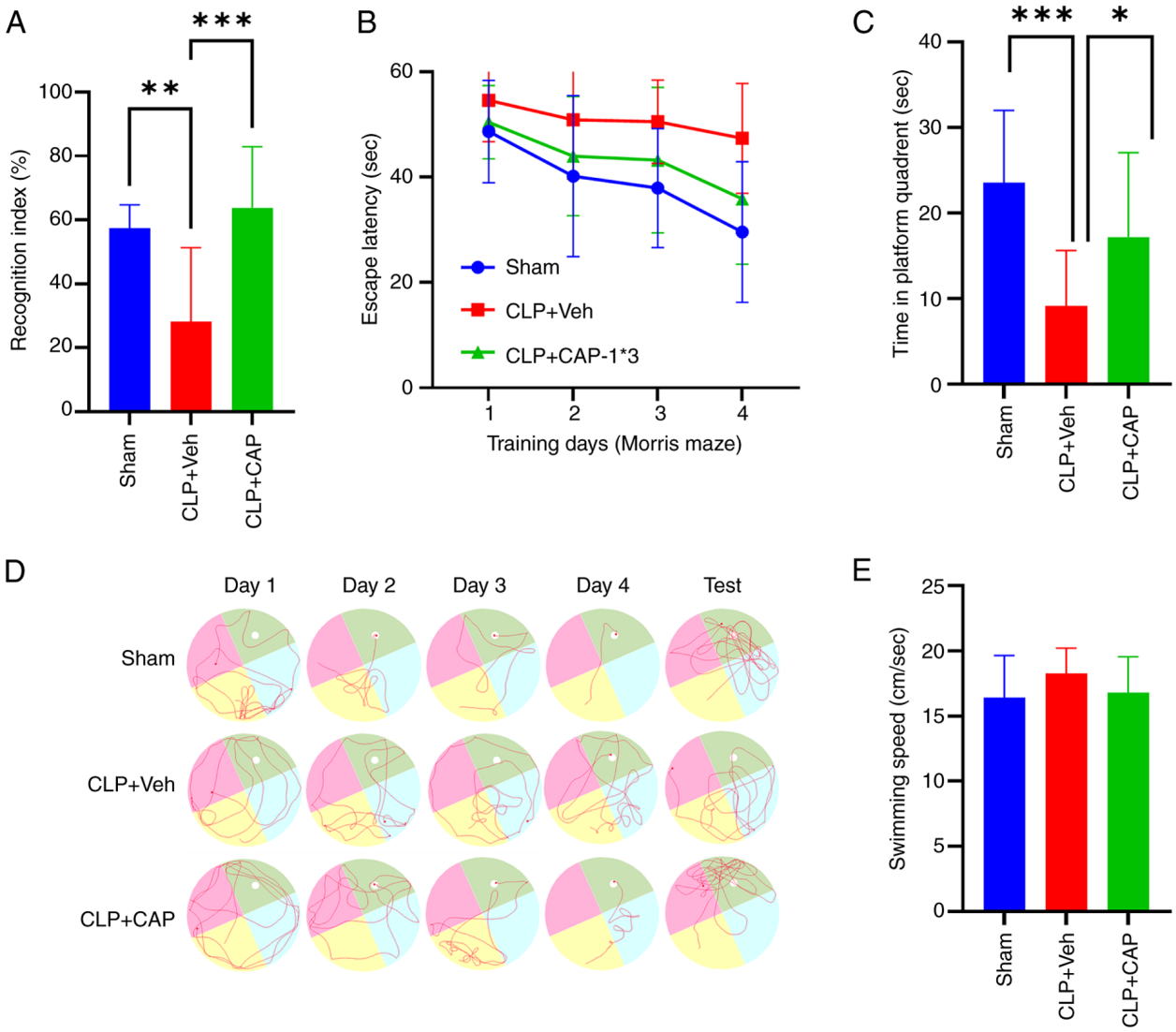

Figure 2. CAP-induced protection in mice with sepsis. (A) Recognition index of the novel object in the novel object recognition test [Sham (n=10), CLP (n=12) and CLP + CAP (n=11) mice; n=10-12 mice/group; one-way ANOVA with Bonferroni post hoc test; $F_{(2,30)}$=12.45; P=0.0001]. (B) Escape latency in the MWM during the training phase [Sham (n=10), CLP (n=15) and CLP + CAP (n=14) mice]. (C) Time spent in the target quadrant in the MWM during the test phase (one-way ANOVA with Bonferroni post hoc test; $F_{(2,35)}$=8.925; P=0.0007). (D) Representative swimming traces in the MWM during the learning and test phase. (E) Average swimming speed in the MWM during the test phase. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001. CAP, capsaicin; CLP, cecal ligation and puncture; MWM, Morris water maze; Veh, vehicle; CAP-1*3, capsaicin 1 mg/kg * 3 application.

group did not exhibit improvements in learning and memory (Fig. 1D).

The effects of s.c. capsaicin administration for 2 days, 1 day and 1 h before CLP surgery on at 1 mg/kg were then assessed on cognitive and memory deficits by NORT and MWM experiments. Similar with single administration, 3 days of application also ameliorated cognitive deficits in mice with sepsis. Specifically, the results of NORT indicated that mice with sepsis exhibited a diminished recognition index compared with those in the sham group, whereas capsaicin application significantly reversed this in the CLP mouse model (Fig. 2A). Mice in the CLP group also had a longer escape latency compared with those in the sham group during the training phase of MWM (Fig. 2B and D). However, the mice that underwent CLP and were treated with capsaicin displayed a significantly reduced escape latency compared with that in the CLP group. In addition, during the testing phase of MWM, mice in the CLP group spent less time in the target quadrant, whereas capsaicin treatment markedly increased the time in this quadrant (Fig. 2C and D). Notably, there was no significant difference in the swim speed among the three treatment groups (Fig. 2E). Taken together, these results suggested that longer-term capsaicin application may ameliorate learning and memory deficits in mice with sepsis, and this dose (1 mg/kg; 3 times) was subsequently used in the following studies.

Capsaicin application suppresses neuroinflammation and neuronal apoptosis in mice following CLP. Subsequently, the extent of microglial activation was assessed. Immunofluorescence staining revealed a notable activation of microglia in the mouse hippocampal tissue following CLP surgery (Fig. 3A-C). The number of Iba1-positive microglia was significantly increased compared with that in the sham

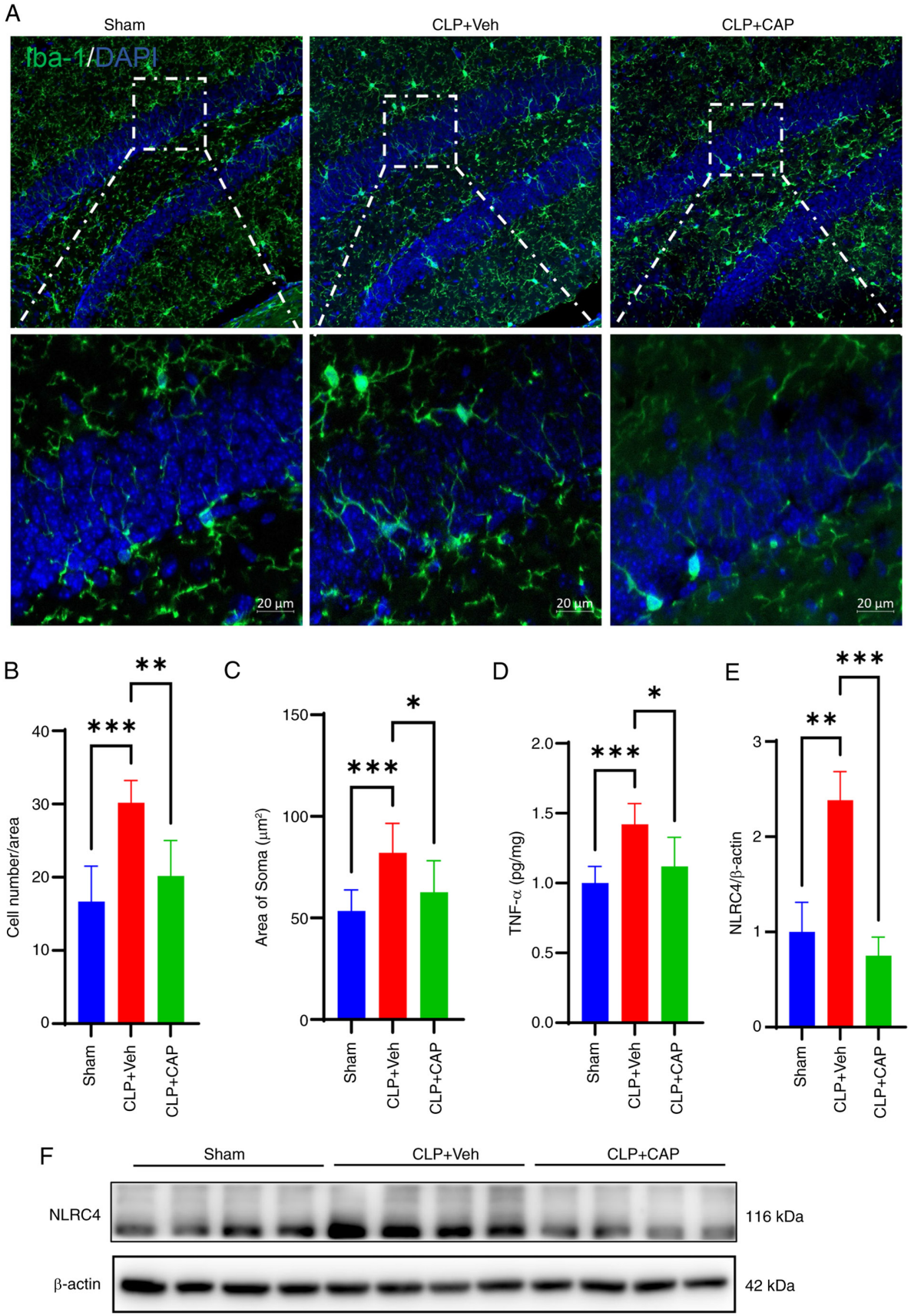

Figure 3. CAP inhibits CLP-induced microglia activation and level of pro-inflammatory factors. (A) Immunofluorescence images of Iba1 (green) fluorescent signals. Scale bars, 10 μm. (B) Quantification of microglia cell numbers (n=6 mice/group) (one-way ANOVA with Bonferroni post hoc test; $F_{(2,15)}$=15.72; P=0.0002). (C) Quantification of the microglial cell area (n=6 mice/group) (one-way ANOVA with Bonferroni post hoc test; $F_{(2,24)}$=10.48; P=0.0005). (D) Levels of TNF-α in the hippocampus (n=6 mice/group) (one-way ANOVA with Bonferroni post hoc test; $F_{(2,15)}$=10.73; P=0.0013). (E) Relative density of NLRC4 expression. (n=8 mice/group) (one-way ANOVA with Bonferroni post hoc test; $F_{(2,21)}$=10.37; P=0.0007). (F) NLRC4 expression was detected by western blotting. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001. CAP, capsaicin; CLP, cecal ligation and puncture; Iba1, ionized calcium-binding adapter molecule 1; NLRC4, nucleotide-binding oligomerization domain, leucine rich repeat and CARD domain-containing 4; Veh, vehicle.

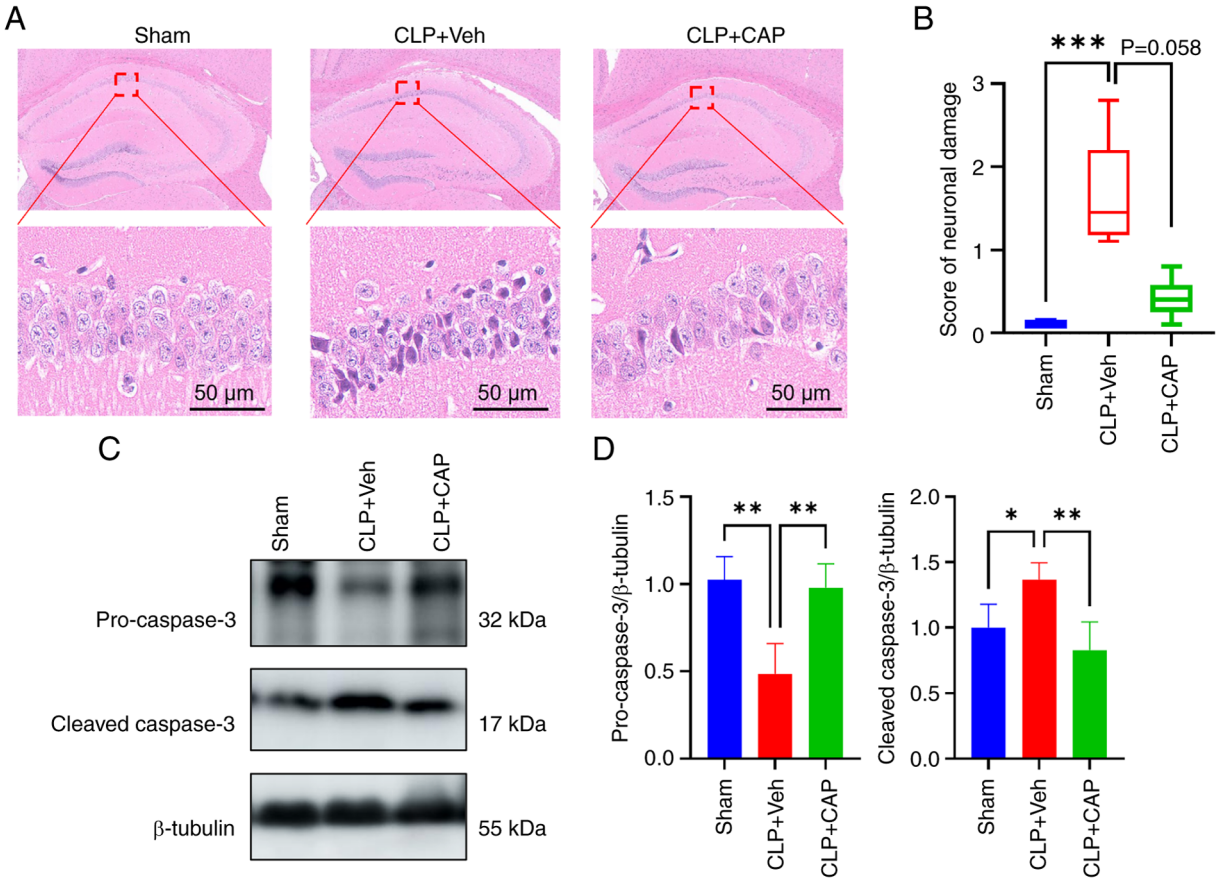

Figure 4. CAP inhibits CLP-induced neuronal damage in mice with sepsis. (A) Representative image of hematoxylin and eosin staining showing the morphological features of neurons in the hippocampal CA1 region. Scale bars, 50 μm. (B) Qualitative analysis of neuronal damage of the hippocampus in septic mice, n=6 mice/group. ***P<0.001, Kruskal-Wallis test with Dunn's post-hoc analysis. (C) Pro-caspase 3 and cleaved caspase 3 expression was detected by western blotting. (D) Relative density of pro-caspase 3 and cleaved caspase 3 expression (n=4 mice/group) (one-way ANOVA with Bonferroni post hoc test; pro-caspase 3: $F_{(2,9)}$=16; P=0.0010; cleaved caspase 3: $F_{(2,9)}$=9.571; P=0.0059). Data are presented as the mean ± standard deviation. *P<0.05 and **P<0.01. CAP, capsaicin; CLP, cecal ligation and puncture; Veh, vehicle.

group, whereas this was reversed by capsaicin application. The levels of TNF-α were detected by ELISA and were revealed to be elevated in mice with sepsis compared with those in the sham group mice; however, this was significantly reversed by capsaicin treatment (Fig. 3D). In addition, the protein expression levels of NLRC4 in the hippocampus were detected and were significantly increased in mice in the CLP group compared with those in the sham-operated group; however, capsaicin treatment reversed this increase (Fig. 3E and F).

To detect the effects of capsaicin on sepsis-induced neuronal damage, H&E staining was performed. The cells in the CA1 region of the hippocampus of mice in the sham-operated group exhibited intact and regular morphology, with clearly delineated boundaries (Fig. 4A). However, the cells in the CLP mice exhibited atrophy and profound staining. Administration of capsaicin resulted in a reduction in the number of abnormal neurons and a decrease in neuronal damage scores (Fig. 4A and B). Next, the protein levels of pro-caspase 3 and cleaved caspase 3 were examined. The expression levels of pro-caspase 3 and cleaved caspase 3 were markedly reduced and enhanced in in the CLP group, respectively, when compared with those in the sham group; however these findings were markedly reserved by capsaicin application (Fig. 4C and D).

Capsaicin application promotes mitophagy through activating BNIP3/NIX-mediated mitophagy. The expression levels of LC3 and p62 were next assessed by western blotting. The results showed that the ratio of LC3-II/LC3-I and p62 expression were significantly increased in the CLP group compared with those in the sham group, whereas capsaicin treatment markedly enhanced the ratio of LC3-II/LC3-I and decreased p62 expression (Fig. 5A-C). TEM was next used to further investigate the degree of mitophagy, which. showed that the number of autophagic vesicles was increased in the mitochondria of the CLP group, whereas the number of autophagic vesicles was further increased in the CLP + capsaicin group compared with that in the CLP group (Fig. 5D).

The observed increase in both p62 expression, an autophagy substrate, and LC3-II induced by CLP indicates a blockage in autophagic flux. Therefore, autophagic flux was next measured using the LC3-GFP-mCherry fluorescent reporter method *in vitro*. As shown in Fig. 6, the relative quantities of yellow puncta were significantly increased in the LPS group, indicating that autophagic flux was blocked; however, in the LPS + capsaicin group, the red puncta were markedly increased, suggesting that autophagic flux was activated. Taken together, these findings suggested that capsaicin application may promote mitophagy.

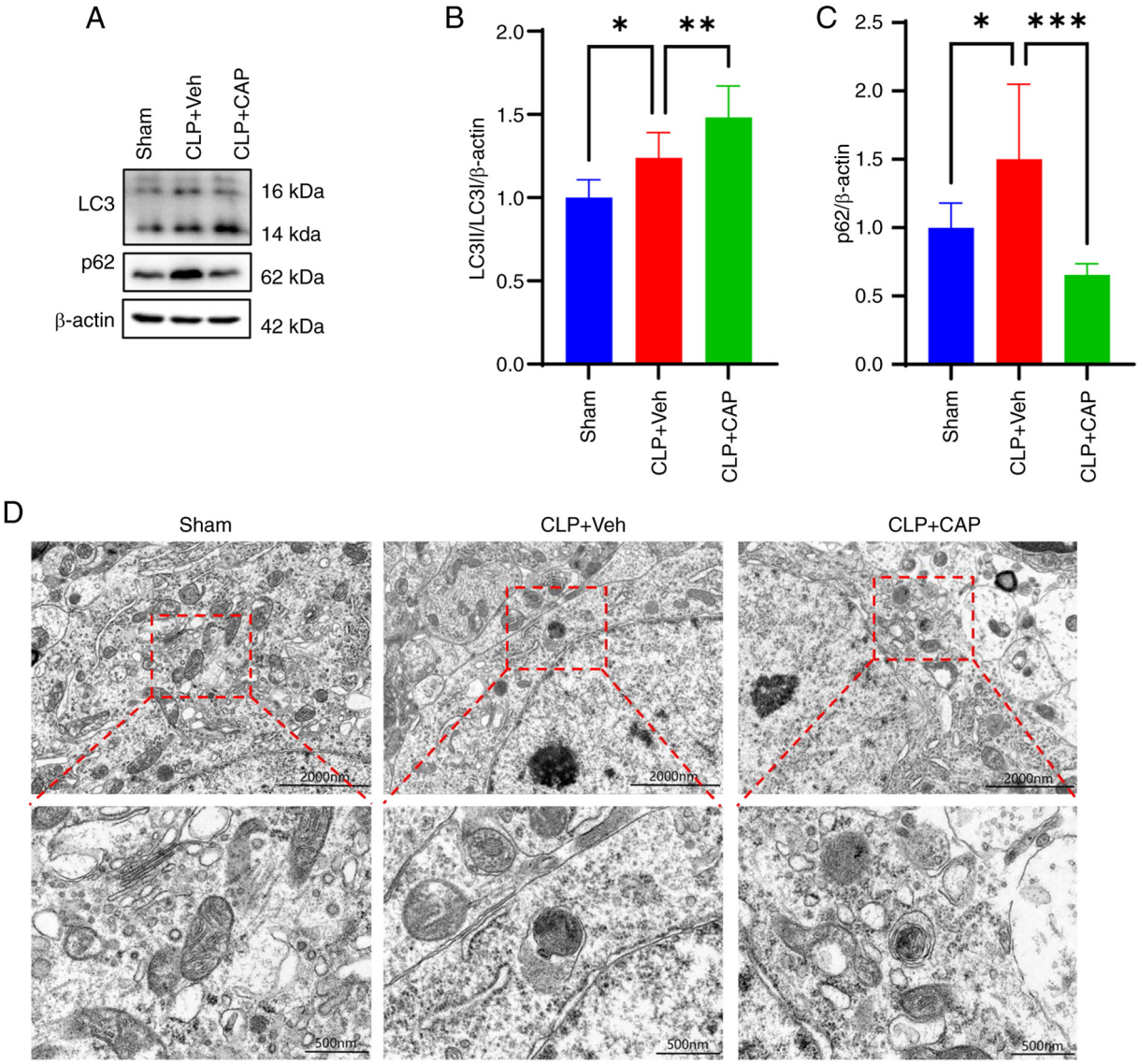

Figure 5. Autophagy level in mice with CLP-induced sepsis. (A) LC3 and p62 protein expression levels in the hippocampus were detected by western blotting. Relative density of (B) LC3 and (C) p62 expression (n=8 mice/group) (one-way ANOVA with Bonferroni post hoc test; LC3: $F_{(2,21)}$=20; P<0.0001; p62: $F_{(2,21)}$=12.76; P=0.0002). (D) Mitophagy was observed under a transmission electron microscope. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001. CAP, capsaicin; CLP, cecal ligation and puncture; Veh, vehicle.

To provide further insights into the molecular mechanisms through which capsaicin can promote mitophagy, the expression levels of proteins associated with this process were assessed. The results revealed that there were no significant changes in the expression levels of PINK1 and Parkin (Fig. 7A-C), whereas BNIP3 and NIX expression were significantly increased after CLP surgery compared with that in the sham group (Fig. 7A, D and E). Notably, capsaicin application further enhanced the expression levels of BNIP3 and NIX induced by CLP. These findings suggested that capsaicin acted through the BNIP3/NIX signaling pathway to activate mitophagy in CLP-induced mice.

Discussion

The present study investigated the role of capsaicin in SAE. Initially, the expression levels of TRPV1 in mice following CLP were shown to be significantly increased. Subsequently, s.c. injection of capsaicin at an appropriate dose (1 mg/kg; 3 times), revealed that a single injection was ineffective. This study demonstrated that three doses, however, significantly improved survival rate, alleviated cognitive dysfunction and promoted hippocampal mitophagy, by increasing autophagic flux. In addition, hippocampal microglia were observed to be activated in mice with SAE (assumed when mice exhibited cognitive impairment in behavioral tests), whereas capsaicin administration reduced microglial activation, release of the proinflammatory cytokine TNF-α, expression of the apoptosis-related protein cleaved caspase 3 and brain tissue destruction, thereby exerting a neuroprotective effect overall. Therefore, capsaicin may protect against SAE by regulating BNIP3/NIX-mediated mitophagy. To the best of our knowledge, the present study was the first to reveal the neuroprotective effects of capsaicin against SAE.

The development of multiorgan dysfunction is the predominant clinical event in sepsis, because of its association with patient morbidity and mortality. Sepsis-induced brain

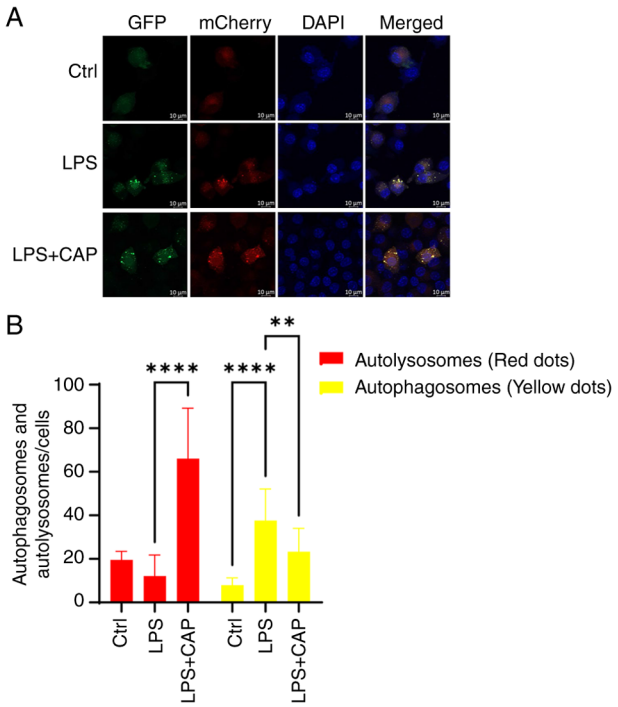

Figure 6. CAP enhances autophagy in LPS-treated BV2 cells. (A) BV2 cells were transfected with LC3-GFP-mCherry. The fluorescence images were captured by confocal microscopy. (B) Quantification of autophagosomes (yellow) and autolysosomes (red) from BV2 cells (one-way ANOVA with Bonferroni post hoc test; $F_{(5,90)}$=44.77; P<0.0001). Data are presented as the mean ± standard deviation. **P<0.01 and ****P<0.0001. CAP, capsaicin; Ctrl, control; LPS, lipopolysaccharide.

dysfunction is a prevalent condition that manifests in the early stages (39). Sepsis has been demonstrated to result in long-term cognitive or emotional impairment, in addition to an elevated risk of mortality (9,40). The CLP mouse model used in the present study has been shown to mimic cognitive dysfunction in previous studies (16,41,42). The presence of long-term cognitive impairment, induced by sepsis, has been observed to be associated with neuroinflammation and pathological tissue changes (43). In the present study, neuroinflammation, apoptosis and brain destruction in the hippocampal brain region were detected, which is consistent with previous studies (44,45).

Previous studies have largely focused on the effects of antagonists, agonists or TRPV1 gene manipulation in the context of sepsis (46,47). However, to the best of our knowledge, only a limited number of studies have examined TRPV1 expression in peripheral tissues or cells, and there is also a paucity of data regarding the expression and function of TRPV1 in the brain. In the present study, TRPV1 expression was shown to be reduced in the hippocampal tissues of mice with sepsis. In previous studies, capsaicin treatment was reported to confer a protective effect against unfavorable outcomes associated with sepsis (23,48). In the present study, capsaicin was shown to markedly improve the survival rate and alleviate cognitive deficits in mice with sepsis. A comparable protective impact of capsaicin was previously documented in studies examining the effects of LPS or CLP-induced multi-organ damage (26,27,49). Therefore, the present findings provide support for the consideration of capsaicin as a promising therapeutic agent for SAE.

Mitochondrial dysfunction serves an important role in SAE pathogenesis (50). A previous study has shown that mitochondria are damaged in SAE, with the release of large quantities of reactive oxygen species (16). Mitophagy removes damaged mitochondria and maintains mitochondrial mass. Dysfunctional mitophagy has been proposed to lead to the accumulation of pathogenic proteins, which in turn induce neurological diseases (51). In the present study, the expression levels of mitophagy-related proteins, including LC3 and p62, were detected in mice following CLP. LC3 expression was revealed to be upregulated by SAE induction and p62 expression was also upregulated, suggesting that autophagy was disturbed. The occurrence of mitophagy was supported by observations from TEM. Under normal conditions, the autophagy receptor p62 is ubiquitinated and subsequently degraded by lysosomes; however, when autophagic flow is blocked, p62 levels increase (52). In the present study, the autophagic flux was monitored using the LC3-GFP-mCherry fluorescent reporter method, which found that the red dots (autolysosomes) were markedly increased after capsaicin treatment, suggesting that capsaicin can promote mitophagy *in vivo*. The present findings substantiated the impact of capsaicin on mitophagy through *in vitro* cell line investigations.

Mitophagy is mediated by the PINK1/Parkin signaling pathway or mitophagic receptors (53). To date, the majority of previous studies have centered on PINK1/Parkin-mediated mitophagy and its role in the clearance of depolarized mitochondria (18,54). The present findings indicated that the PINK1/Parkin pathway was not implicated in the protective effects of capsaicin on SAE. Instead, BNIP/NIX-mediated mitophagy more likely served the important role in this process. At present, it remains unclear whether capsaicin can regulate BNIP3/NIX through TRPV1 channels. In addition, to the best of our knowledge, no studies have explored the potential interaction between TRPV1 and BNIP3/NIX. Therefore, it is necessary to further study the component immediately downstream of capsaicin.

Notably, there are limitations in the present study that must be acknowledged. The present study does not exclude the notion that capsaicin can exert an ameliorative effect on SAE through TRPV1 in a non-dependent manner. Therefore, TRPV1 knockout mice should be used in the future. The present study also exclusively utilized 8-10-week-old male mice, neglecting the potential variations in sex and age. Previous studies have demonstrated that male patients afflicted with sepsis exhibit an elevated mortality rate, whereas individuals aged ≥65 years also demonstrate a 13x increased likelihood of developing sepsis and encountering a higher mortality rate (55,56). Consequently, it is imperative that future studies take into account the effects of both sex and age on the outcomes under investigation. In addition, in the present study, the mechanism through which capsaicin can promote the BNIP3/NIX pathway was not explored. In the future, the interaction between TRPV1 and BNIP3/NIX or the binding domains in which capsaicin acts with BNIP3/NIX should be investigated.

In conclusion, the present study highlighted the role of capsaicin in SAE. Capsaicin application could effectively improve the survival rate of septic mice, improved cognitive function, reduced neuroinflammation and apoptosis, and promoted mitophagy. The mechanism of protection may involve BNIP3-NIX-mediated mitophagy. Capsaicin may be as a novel treatment strategy for SAE.

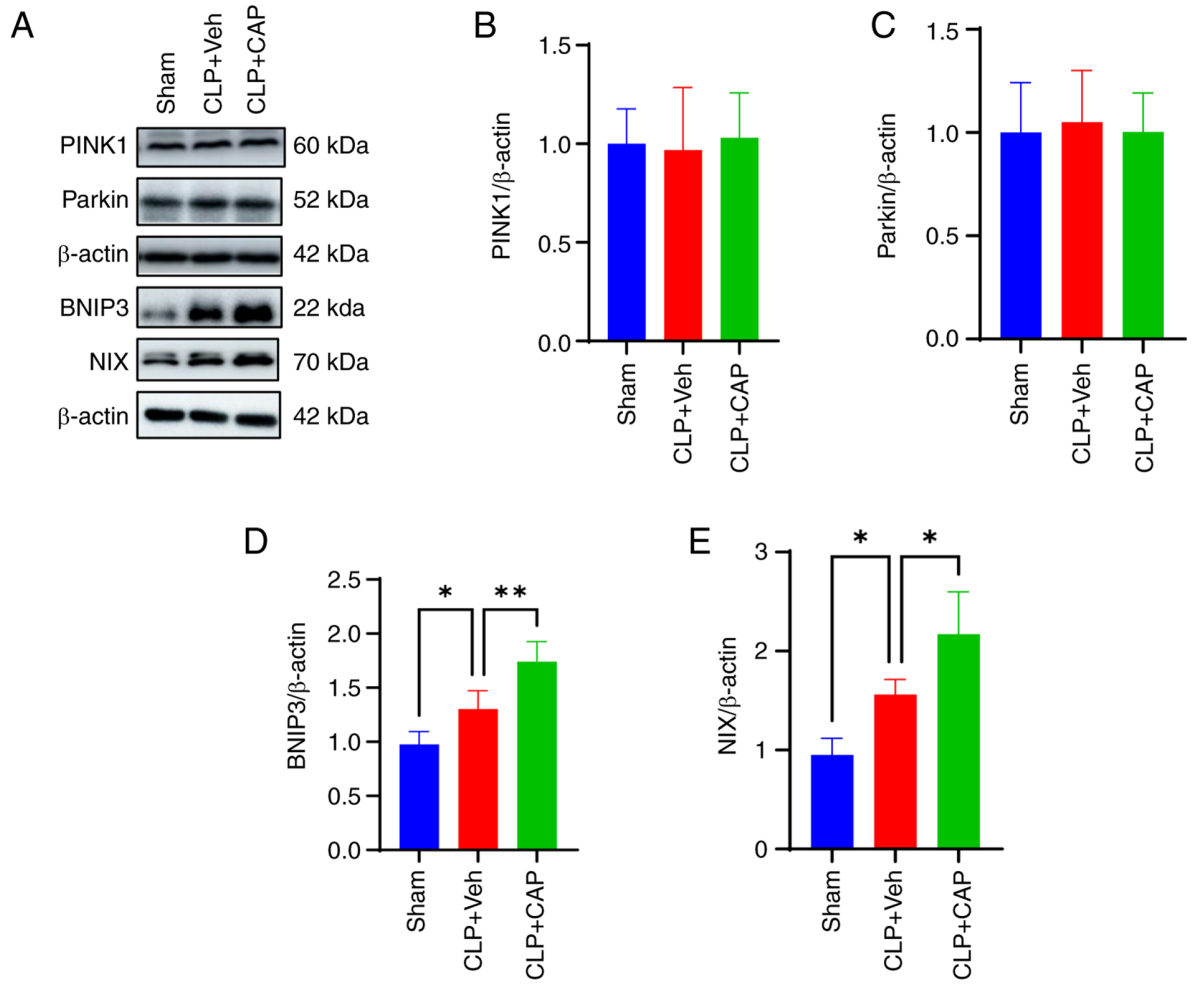

Figure 7. Capsaicin promotes mitophagy via the BNIP3/NIX pathway. (A) PINK1, Parkin, BNIP3 and NIX proteins were detected by western blotting. Relative density of (B) PINK1, (C) Parkin, (D) BNIP3 and (E) NIX expression; n=4 mice/group. (one-way ANOVA with Bonferroni post hoc test; BNIP3: $F_{(2,9)}$=22.79; P=0.0003; NIX: $F_{(2,9)}$=22; P=0.0003). Data are presented as the mean ± standard deviation. *P<0.05 and **P<0.01. BNIP3, Bcl-2-interacting protein 3; CAP, capsaicin; CLP, cecal ligation and puncture; NIX, BNIP3-like; PINK1, PTEN-induced kinase 1; Veh, vehicle.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YLi performed and analyzed the study. YLi and SZ constructed figures. NL and SZ performed the experiments. HW contributed to the establishment of the sepsis model. HW and SZ performed and analyzed the western blotting and ELISA. JC and YJ contributed to cell culture. SZ performed the immunofluorescence staining. LX and HL analyzed the data. YLiu and SZ wrote the paper. YLi, QX, YLu, BY and ZL designed the study. YLi and ZL supervised the research. NL and SZ confirm the authenticity of all the raw data. YLi, QX and ZL acquired the funding. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal study was reviewed and approved by the Committee on the Animal Research Ethics of the China Technology Industry Holdings (Shenzhen) Co., Ltd. (approval no. 202200105).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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