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# Umbilical Cord Mesenchymal Stem Cells Limit Post-Stroke Infection

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## Abstract

Brain ischemia leads to excessive infiltration of clusters of $CD8^+$ T and natural killer (NK) cells in the brain, which aggravate ischemic brain injury. Acute ischemic stroke also has a negative impact on the antibacterial immune response, leading to stroke-induced immunodepression and infection. Umbilical cord mesenchymal stem cell (UCMSC) have an immunosupn pressive function. Therefore, we aimed to determine whether UCMSC treatment alleviates the excessive infiltration of $CD8^+$ T and NK cells. We also investigated significant concerns that UCMSC treatment might suppress antimicrobial immunity, leading to an increased risk of infection.In this study,stroke and post-stroke infective mice received intravenous injection of UCMSC. We found UCMSC treatment ameliorated the infiltration of $CD8^+$ T and NK cells in the brain, reduced levels of proinflammatory cytokines, and increased anti-inflammatory cytokines.UCMSC treatment limit post-stroke infection and reduce the inflmamatory injury of various organs induced by post-stroke infection.What's more ,UCMSC treatment maintained autophagy, MMP, and the production of ATP, while inhibiting apoptosis of platelets caused by post-stroke infection in vivo.Then we also found UCMSC enhance the antibacterial ability of platelets in vitro,implying that UCMSC can limit post-stroke infection partly via the regulation of platelets.Based on these findings, UCMSC represent a potential and safe therapeutic option for stroke treatment by inhibiting brain injury and limiting post-stroke infection.

**Keywords**: Post-Stroke Infection; Platelet

**List of abbreviations:** UCMSC: umbilical cord mesenchymal stem cell; IL: interleukin; MCAO: middle cerebral artery occlusion; mNSS: modified neurological severity score; NK: natural killer cell; AST: aspartate aminotransferase; ALT: alanine aminotransferase; LDH: lactate dehydrogenase; CK: creatine kinase; MMP: mitochondrial membrane potential; LB: Luria-Bertani

## Introduction

Infectious complications—primarily pneumonia and urinary tract infection—are a leading cause of death in ischemic stroke patients[1,2].The impairment of immune responses after brain ischemia increases susceptibility to infections[3,4]. Excessive infiltration of cluster of differentiation (CD)8+ T cells or natural killer (NK) cells in the brain aggravates ischemic brain injury, while brain ischemia compromises NK cell-mediated immune defence in the periphery and can result in post-stroke infection [5-7].In addition,the grade of immunoinflammatory activation could be related to pathogenesis of neuronal damage in ischemic stroke [8,9]. For example,TNF-α (Tumor necrosis factor-α) and IL-6 (Interleukin-6) express a higher level in plasma of acute ischemic stroke patients,which play a pivotal role in inflammatory processes that aggravate ischemic neural damage [10]. MSCs have been shown to attenuate harmful immune responses locally and systematically, providing a significant degree of neuroprotection. Careful attenuation of the immune response after stroke affords neuroprotective effects with stem cells playing an active role as potent mediator of immunomodulation, which is one of the principal mechanisms by which they exert a neuroprotective effect [11]. However, it is not completely clear how stem cell therapy regulates the immune system, and this effect may be multifactorial in nature. While mesenchymal stem cells (MSCs) have been shown to exert therapeutic effects following stroke, inhibit the proliferation and effector functions of various immune cells, including T and B lymphocytes and NK cells [12-17]. So we remain concerned that MSC transplantation may also suppress antimicrobial immunity and increase the risk of post-stroke infection.

In our study, we first detected no obvious signs of infection in mice with stroke with or without umbilical cord (uc)MSC treatment. In another study, however, we established a post-stroke infection model using the gram-positive intracellular bacteria Listeria monocytogenes [6] or Escherichia coli, and this decreased the number of platelets in mice, while UCMSC treatment reversed this change. Symptoms of infection were also alleviated when these mice were injected with UCMSC, suggesting their therapeutic potential against post-stroke infection. This potential was investigated further in the present study by examining the effects of UCMSC on platelets and post-stroke infection, as well as determining the underlying mechanisms for these effects.

## Methods

### Animals

C57BL6 mice were purchased from the Laboratory Animal Center of Southern Medical University and were maintained under standard laboratory conditions, with the temperature controlled at 24 °C and with free access to a standard diet and sterile water. All animal procedures were performed in accordance with the guidelines and approval of the Animal Ethics Committee of Southern Medical University (ethics approval code:2017-SJWK-009).

### Middle cerebral artery occlusion (MCAO) and post-stroke infection model

Mice weighing 20–22 g (aged 7–8 weeks) were allowed free access to water but were fasted for 12 h to standardize glycaemic state. MCAO was performed under anaesthesia induced by intraperitoneal injection of pentobarbital (100 mg/kg). Body temperature was maintained at 37°C ± 0.5°C using a heating pad (RWD Life Science, Shenzhen, China). To induce MCAO, a 6-0 nylon suture (Covidien, Mansfield, MA, USA) with a round tip and silicon coating was inserted from the left external carotid artery into the middle cerebral artery. The success of the surgery was verified by monitoring surface cerebral blood flow using a laser Doppler flowmeter (Moor Instruments, Devon, UK). After 1 h, the occluding filament was gently withdrawn back into the common carotid artery to allow reperfusion. Mice in the sham group underwent a sham operation without suture insertion.

*E. coli* were cultured as previously described [18] and stored in 30% glycerol at −80°C until use. *E. coli* were grown in Luria-Bertani (LB) medium (10 g/l tryptone, 5 g/l yeast extract, and 171.1 mM NaCl). Growth was determined by measuring the optical density at 620 nm ($OD_{620}$) or by plating the cells on LB plates and counting viable cells. For infection, age-matched male mice were intravenously injected with $10^7$ colony forming units (CFU) of *E. coli* resuspended in 500 µl phosphate-buffered saline (PBS) immediately after sham or MCAO operation.To determine the degree of infection, the mouse liver, lung, and brain were removed and homogenized in distilled water with 0.01% Triton X-100. The number of viable *E. coli* cells was counted after plating serial dilutions of organ homogenates and blood on LB plates and culturing overnight at 37 °C.

### UCMSC culture and transplantation

The 1-2 passage UCMSCs were kindly presented by

Guangzhou SALIAI Stemcell Science and Technology Co.,Ltd. The cells were supplemented with special culture medium for UCMSCs, and grown at 37°C in a humidified 5% CO2 atmosphere. The culture medium was changed every 2- 3 days, and the cells were passaged at approximately 80% confluence.The mice were randomly divided into the following different groups (n = 5 mice per group) after MCAO: Sham, MCAO , MCAO + UCMSCs OR CTRL, Sham+Ecoi,MCAO+Ecoi ,MCAO+UCMSCs+Ecoi . The transplantation of 3-5 passage UCMSCs through caudal vein injection ($10^6$ cells in 0.5 mL PBS) was perf ormed 2 h after transient MCAO or MCAO+Ecoi. The same amount of PBS without cells was injected as the control.

Assessment of neurological function and measurement of cerebral infarct area

Neurological function was determined based on the Modified Neurological Severity Score (mNSS). The mNSS test consists of ten different tasks that can evaluate the motor (muscle status, abnormal movement), sensory (visual, tactile and proprioceptive), balance, and reflex functions of mice. Neurological function was graded from 0 to 18 (0 = normal function; 18 = maximal deficit). One point was scored for each abnormal behavior or for the lack of a tested reflex. Therefore, higher scores implying greater neurological injury[19].The test was carried out by a blinded investigator before and 3 days after MCAO, as previously described[20]. The infarct areas of different experimental groups were measured in photomicrographs of methylthioninium chloride-stained tissue sections (5 sections/animal). Experiments were repeated five times.

Hematoxylin and eosin (HE) staining, immunohistochemistry, immunofluorescence analysis and flow cytometry analysis

At 24 h after MCAO or post-stroke infection, mice were anesthetized and transcardially perfused with 20 ml cold PBS and 20 ml of 4% paraformaldehyde in 0.1 M PBS. The brain, lung, liver, and spleen were removed, post-fixed, and embedded in paraffin. The tissue blocks were cut into 5-mm sections that were deparaffinized and stained with HE according to standard protocols.

$CD8^+$ T cells and NK cells in the brain and spleen were identified by immunofluorescence analysis and immunohistochemistry, as previously described. For the latter, brain and spleen tissue sections were incubated overnight at 4°C with primary antibodies against CD8 (ab25117) and natural cytotoxicity receptor (NCR) (ab199128), Iba1(ab5076), CD68(ab125212) (Abcam,Cambridge,MA,USA) respectively followed by processing with avidin-biotin-peroxidase (BosterBio, Wuhan, China). The sections were stained with diaminobenzidine, and nuclei were counterstained with hematoxylin.

For immunofluorescence, the specimens were first treated with anti-CD8 or -NCR antibody, followed by Alexa Fluor 594-conjugated secondary antibody (A0453; Beyotime Institute of Biotechnology, Shanghai, China). Immunofluorescence images were acquired with a confocal laser scanning microscope (TCS SP2; Leica Microsystems, Wetzlar, Germany).

## Blood biochemical analysis

Mouse blood was collected via the angular vein under anaesthesia into an anticoagulant-containing tube. Biochemical analyses were performed at Southern Medical University Huayin Laboratory.

## Enzyme-linked immunosorbent assay (ELISA)

Plasma was isolated by centrifugation of blood samples at 1500 rpm for 20 min. TNF-α, IL-6, IL-10 in the plasma were detected with ELISA kits (Cusabio, Wuhan, China) according to the manufacturer's instructions. Briefly, 100 μl of plasma was added to each well of a 96-well plate. After incubation for 2 h at 37 °C, the plasma was removed, and the plates were sequentially incubated with biotin-conjugated primary antibody followed by horseradish peroxidase-conjugated secondary antibody for 1 h at 37°C, with three washes between each step. After adding the chromogenic substrate, the plates were incubated in the dark for 30 min at 37°C. The reaction was terminated, and the $OD_{450}$ was measured using an iMark microplate reader (Bio-Rad, Hercules, CA, USA).

## Co-culture of bacteria and UCMSC

Platelets alone or in combination with UCMSC were inoculated with *E. coli* for 1, 2, 4, or 6 h. Bacterial growth was determined by measuring the $OD_{620}$.

## Statistical analysis

Statistical analysis was performed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Data are presented as the mean ± SD. The significance of differences between means was examined by Student's *t*-test or one-way analysis of variance. Results with $P < 0.05$ were considered significant.

## Results

### UCMSC decrease brain lesion size and improve neurological function after stroke

We evaluated the effect of UCMSC on stroke based on measurement of the lesion area and the mNSS in an MCAO mouse stroke model. mNSS scores ($P < 0.05$; Figure 1A) as well as the lesion area ($P < 0.05$; Figure 1B) were reduced in the UCMSC treatment group as compared to those of the MCAO group, suggesting that UCMSC exert therapeutic effects after stroke.

### UCMSC inhibit immunological function after stroke

We next examined the immunomodulatory effects of UCMSC treatment on the post-stroke brain by examining the abundance of $CD8^+$ T cells and NK cells by immunohistochemistry and immunofluorescence analysis, as well as flow cytometric analysis. Both cell populations were diminished in mice treated with UCMSC as compared to that in the MCAO group (Figure 2A–C). We detected the activated microglia by staining Iba1and CD68 (activated microglia marker) after induction of MCAO. Iba1- and CD68-positive cells were increased after MCAO,while

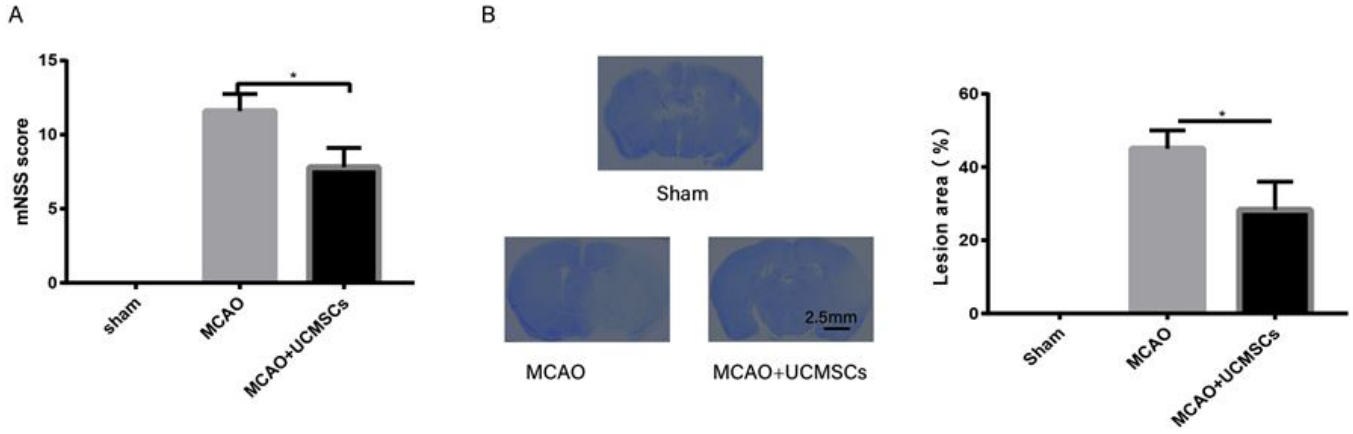


**Figure 1:** The therapeutic effect of UCMSC in MCAO mice. MCAO mice were treated with or without UCMSC. (A) mNSS and (B) lesion area in each group. The data are plotted as the means ± SD. *$P < 0.05$, n=5 or 3.

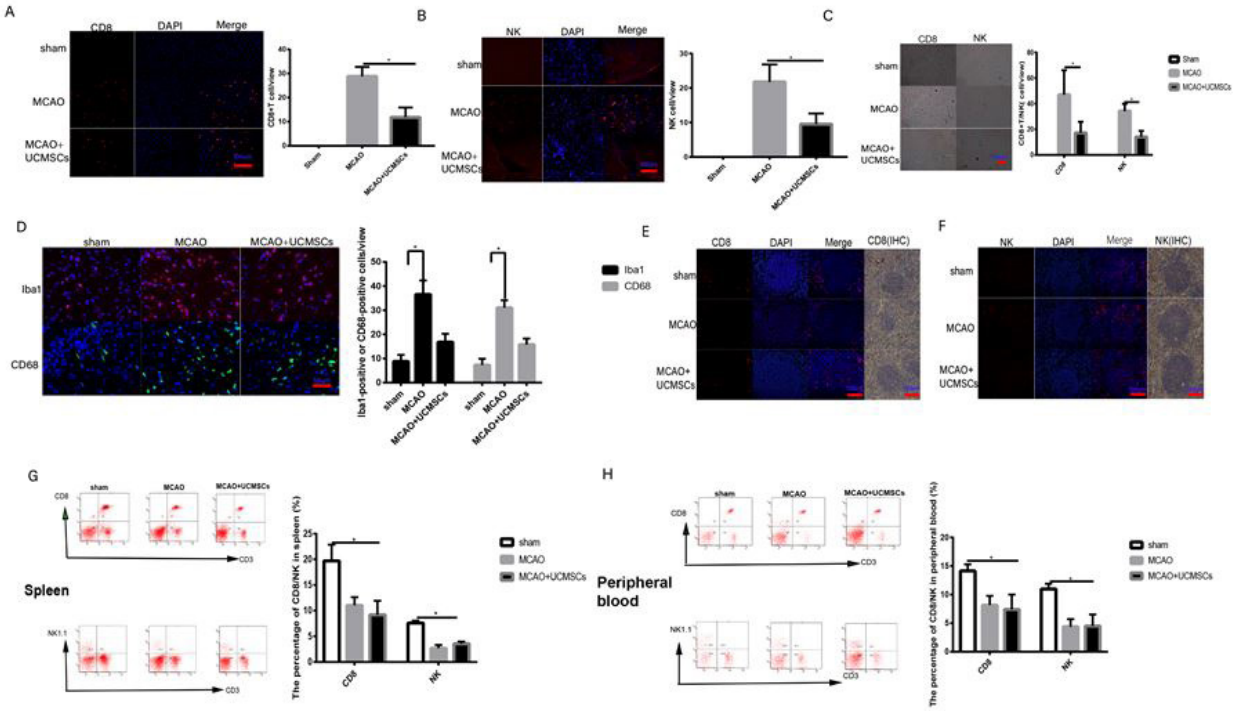


**Figure 2:** UCMSC reduced $CD8^+$ T/NK cells in MCAO mouse brain but not spleen or blood. (A–C) Immunohistochemistry and immunofluorescence of $CD8^+$ T cells and NK cells in the brain. (D) Immunofluorescence of the activated state of microglia.(E–H) Immunofluorescence and flow cytometry of $CD8^+$ T cells and NK cells in the spleen and blood. Scale bar: 100 μm. The data are plotted as the means ± SD. *$P < 0.05$, n=5

UCMSC treatment decreased the Iba1-positive and CD68-positive cells (Figure 2D).Additionally, the proportions of $CD8^+$ T cells and NK cells were decreased in the spleen and peripheral blood after MCAO, but these changes were not abrogated in the MCAO + UCMSC group (Figure 2D–G). Meanwhile, plasma levels of the pro-inflammatory cytokines interleukin (IL)-6 and tumour necrosis factor (TNF)-α were lower, whereas that of the anti-inflammatory cytokine IL-10 was higher, in UCMSC-treated mice as compared to levels in untreated MCAO mice, as determined by ELISA (Figure 3A–C).

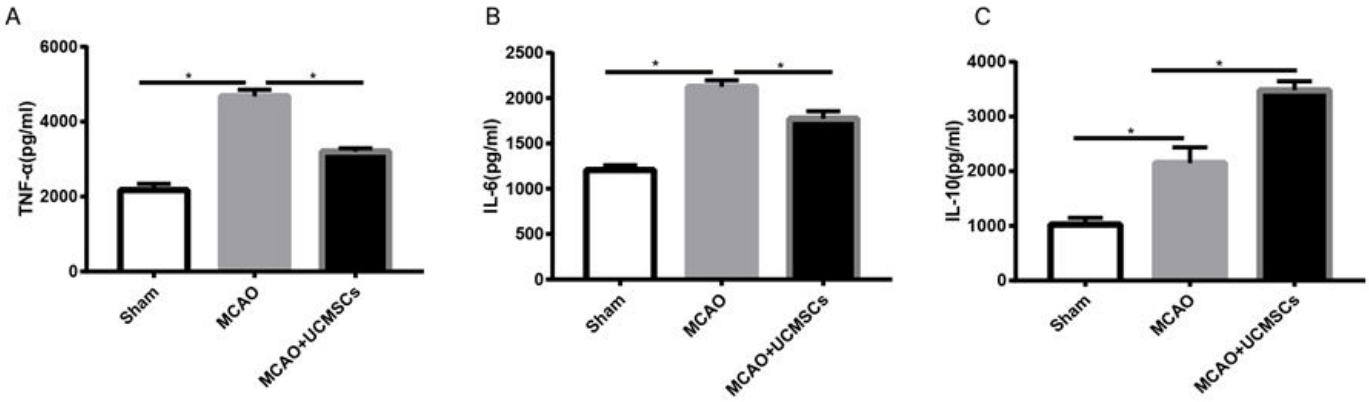


**Figure 3:** Expression of pro-inflammatory cytokines IL-6, TNF-α and IL-10 in mouse plasma. The data are plotted as the means ± SD. *P < 0.05, n=3

### UCMSC treatment mitigates infection after stroke and prevents organ damage

HE staining of lung, liver, and spleen tissue sections as well as blood routine revealed no signs of infection in MCAO mice with or without UCMSC treatment (Figure 4). To assess the effect of systemically administered UCMSC on post-stroke infection, MCAO mice with or without UCMSC treatment and E. coli infection were examined for the presence of bacteria in the brain, lung, liver, and spleen. Compared to the MCAO group, MCAO + UCMSC mice showed a lower bacterial burden in these organs, including a reduction in the size of the germinal centre of the spleen (Figure 5E). During post-stroke infection, inflammatory cells infiltrated the lung, brain, and liver and caused cell and tissue damage; these effects were alleviated by UCMSC treatment. We also measured aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), and lactate dehydrogenase (LDH) levels in plasma and found that AST, ALT, CK and LDH were downregulated in the UCMSC treatment group as compared to levels in the MCAO group during the course of infection. Plasma TNF-α and IL-6 levels were also reduced, whereas IL-10 was upregulated by UCMSC treatment.

### UCMSC and platelets have synergistic antibacterial effect

To investigate whether UCMSC enhance the antibacterial ability of platelets, platelets were co-cultured with E. coli. Finally,we found the growth of E. coli was further inhibited in the presence of UCMSC (Figure 7).

## Discussion

Infection in the lungs and other organs are relatively common during the subacute stage of stroke and are associated with adverse outcomes[21]. Preventative antibiotic therapy does not influence functional outcomes in the overall population [22,23]. Ischemic stroke negatively impacts the antibacterial immune response, leading to stroke-induced immunosuppression and infection[7,24]. For example, brain ischemia can cause a reduction in NK cell numbers and response in the periphery via activation of the catecholaminergic system and hypothalamic-pituitary-adrenal axis, which can result in infectious complications [6]. In accordance with previous studies [25], we observed a decrease in the numbers of $CD8^+$ T cells and NK cells in the spleen and peripheral blood after stroke, whereas more of these cells infiltrated the brain tissue, which could aggravate brain injury. MSCs (mesenchymal stem cells) have immunomodulatory activity and are therefore promising agents for cell-based therapies. MSCs regulate a variety of immune cells—for example, they inhibit the activation and proliferation of T cells and induce T cell apoptosis while suppressing the differentiation and maturation of dendritic cells [26-30]. MSCs have also been shown to

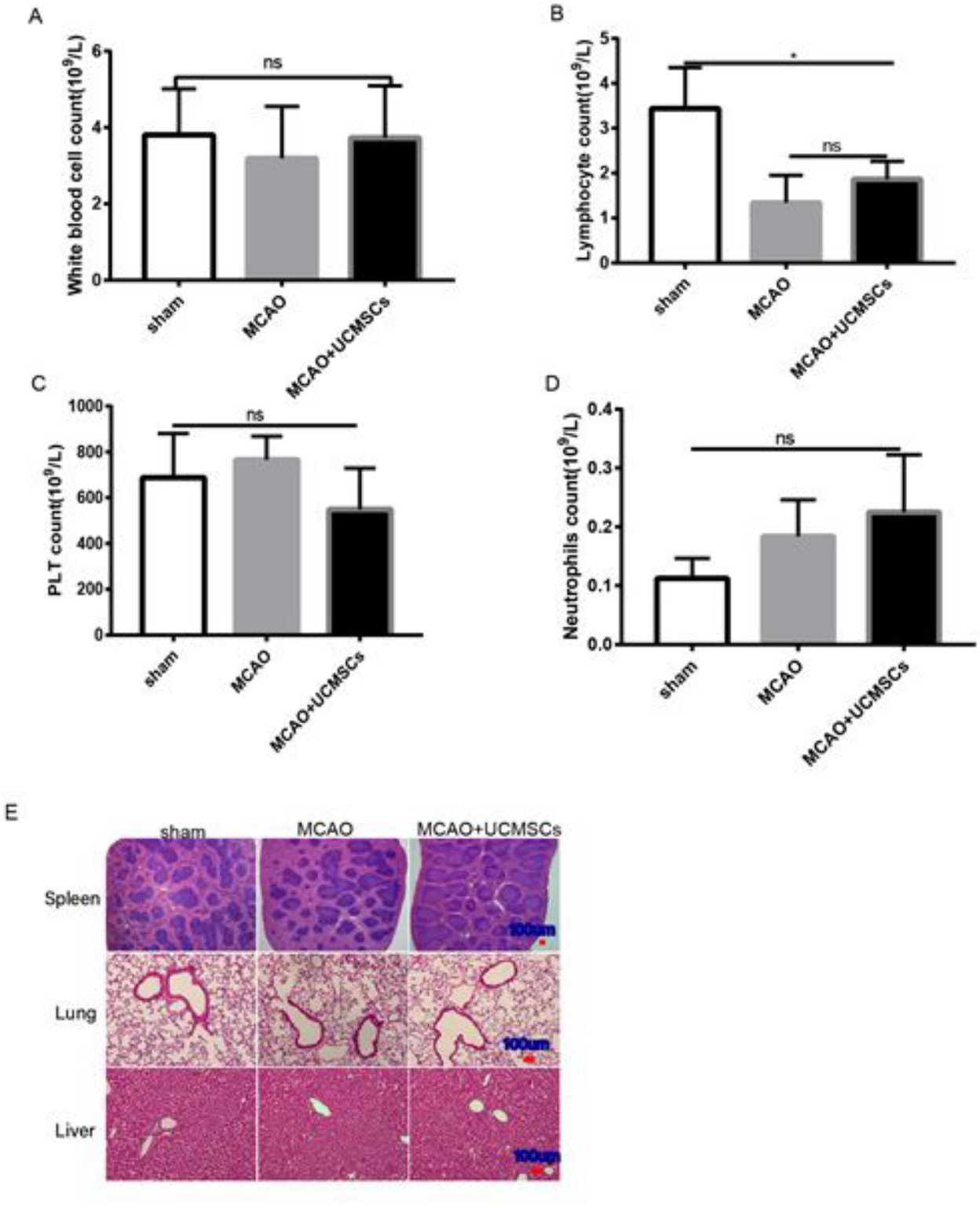


**Figure 4.** No infective signs were found in the MCAO or UCMSC groups. MCAO mice were treated with or without UCMSC, followed by analysis of (A) white blood cells, (B) lymphocytes, (C) neutrophils, and (D) platelets, as well as (E) HE staining of the brain, lung, liver, and spleen. Scale bar: 100 μm. The data are plotted as the means ± SD. NS, not significant. *P < 0.05, n=5

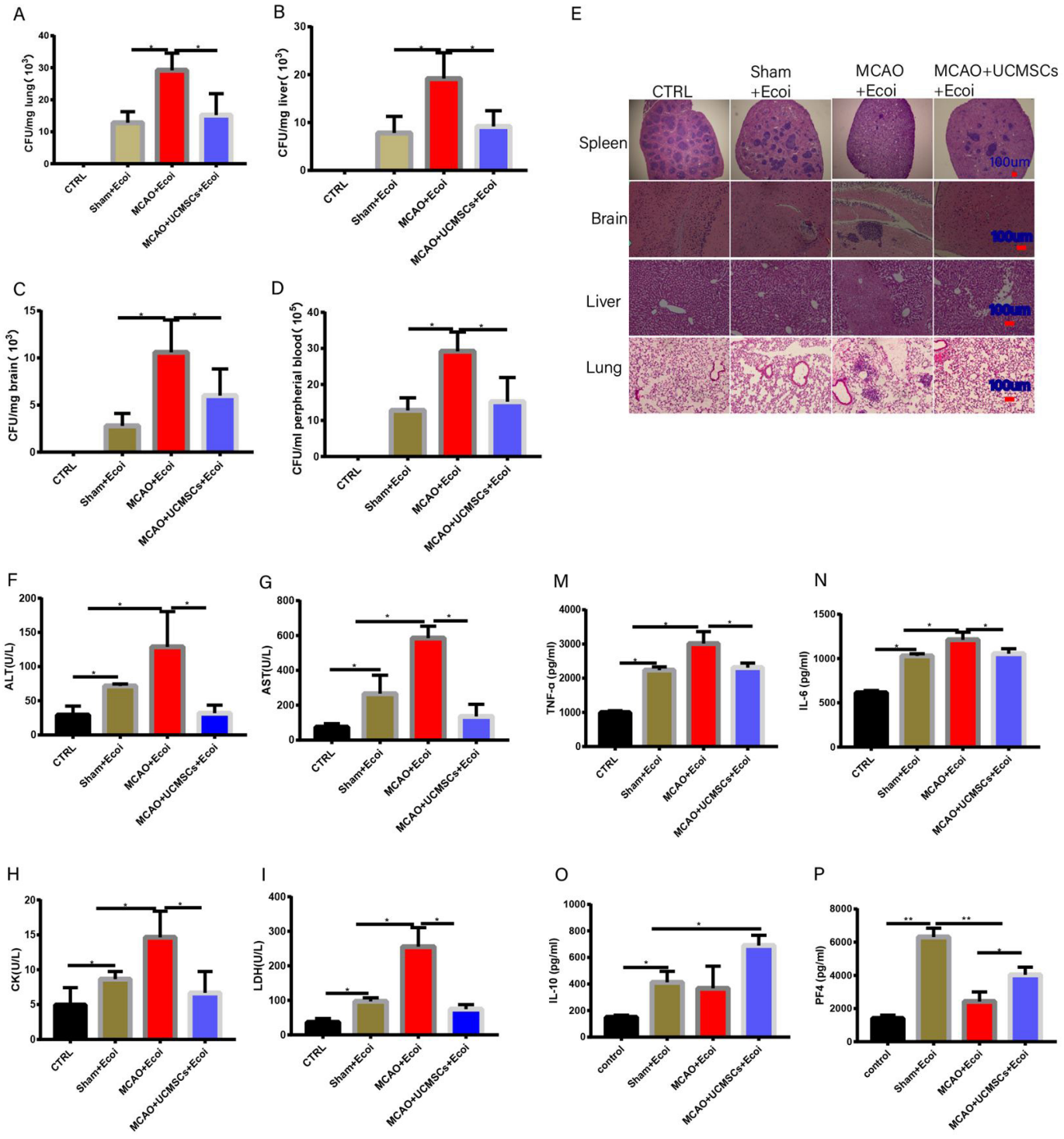


**Figure 5:** UCMSC limit post-stroke infection and protect important organs. MCAO mice were treated with or without UCMSC in the presence or absence of *Escherichia coli*. The bacterial burden was assessed in the (A) lung, (B) liver, (C) brain, and (D) blood. (E) HE staining showing the germinal centre of the spleen and damage in the lung, brain, and liver. Plasma levels of (F) ALT, (G) AST, (I) LDH, (M) TNF-α, (N) IL-6, (O) IL-10. Scale bar: 100 μm. The data are plotted as the means ± SD. **P < 0.01, *P < 0.05, n=5.

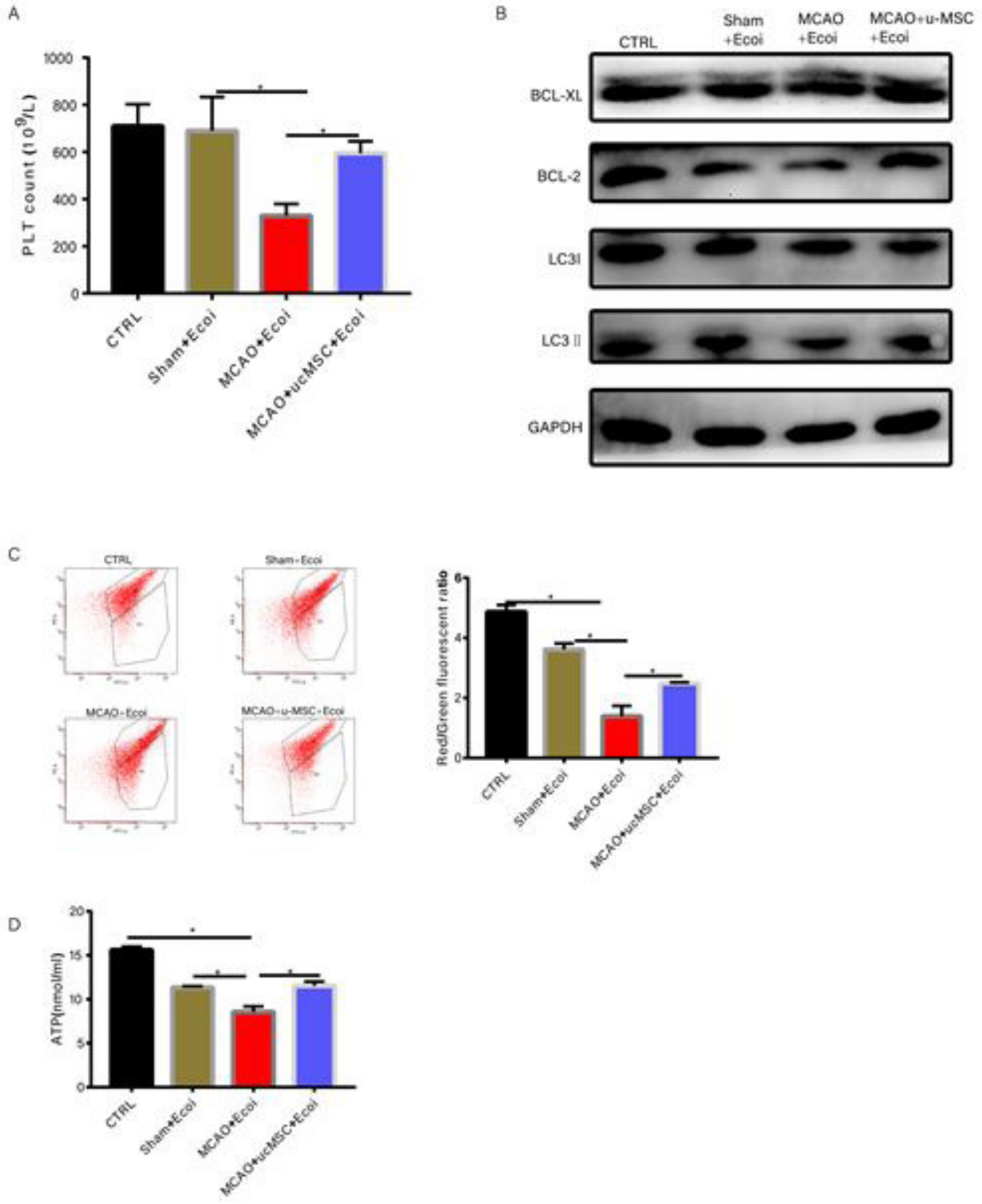


**Figure 6:** Platelet function was maintained by UCMSC treatment *in vivo*. MCAO mice were treated with or without UCMSCs in the presence or absence of *Escherichia coli*. (A) Numbers of platelets. (B) Levels of autophagy marker LC3-II and apoptosis markers Bcl-2 and Bcl-xL. Platelet (C) mitochondrial membrane potential (MMP) and (D) ATP levels. The data are plotted as the means ± SD. *P < 0.05, n=5

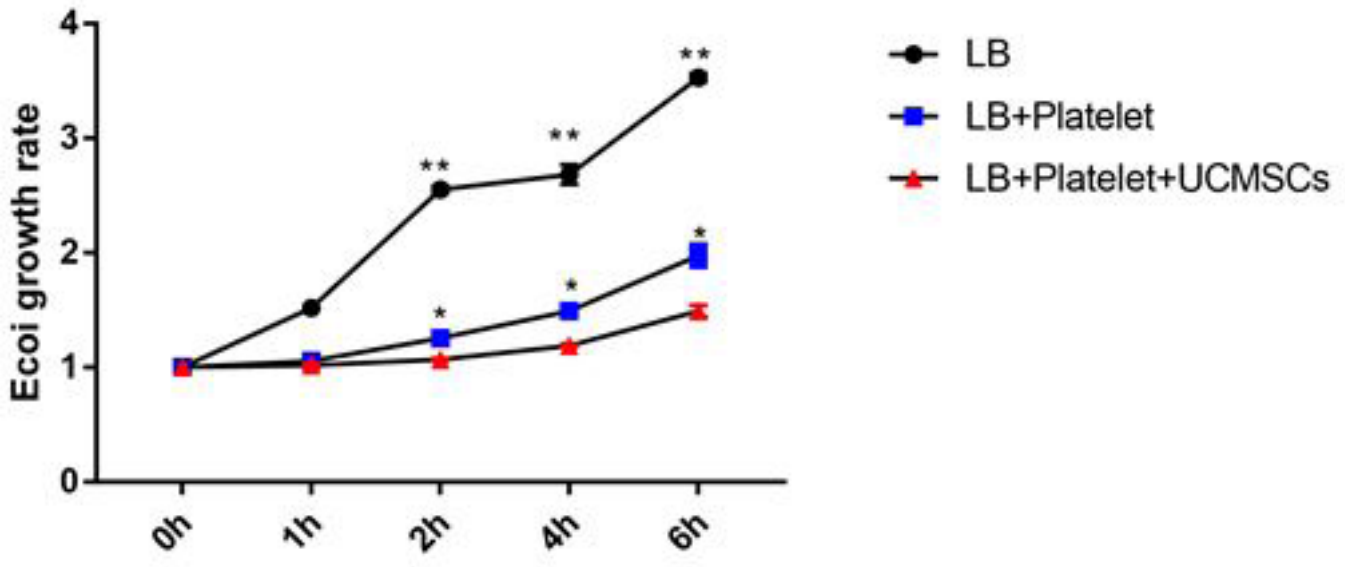


**Figure 7:** Antimicrobial activity of platelets was enhanced by UCMSC. Growth rate of *Escherichia coli* in Luria-Bertani medium co-cultured with or without platelets in the presence or absence of UCMSC. The data are plotted as the means ± SD. **P < 0.01, *P < 0.05, n=5

block NK cell activity [31-33]. In our study, we found that UCMSC reduced the number of CD8+ T cells and NK cells in brain tissue but not in the spleen or peripheral blood of mice following stroke, suggesting that UCMSC can prevent brain injury. Furthermore, plasma levels of the pro-inflammatory cytokines IL-6 and TNF-α were reduced, whereas that of the anti-inflammatory cytokine IL-10 was increased by UCMSC treatment, confirming that UCMSC induce immunosuppression [34].

One point of concern is whether UCMSC can increase the risk of infection after stroke by suppressing antimicrobial immunity. However, symptoms of post-stroke infection were alleviated in mice following UCMSC treatment, which not only inhibited the growth of bacteria in certain organs but also prevented tissue damage caused by bacteria and inflammatory factors. Post-stroke pneumonia is a major cause of death after stroke [35]. In our study, UCMSC treatment reduced haemorrhage, oedema, and cellularity in injured lung lobes caused by E. coli. So, our results show that UCMSC play a protective role against post-stroke infection, but the underlying mechanisms were not completely clear.

Recent data show that MSCs exert strong antimicrobial effects through indirect and direct mechanisms, partially mediated by the secretion of antimicrobial peptides and proteins (AMPs)[36-38],which may be one reason of UCMSC inhibiting the post-stroke infection .However,we also found UCMSC inhibit the apoptosis of platelets,as well as maintain the count of platelets after post-stroke infection.In previous studies, platelets have been shown to inhibit bacterial growth by surrounding bacteria and secreting a high concentration of antimicrobial substances [39]. Platelets also activate some immune cell types to fight bacteria and work with Kupffer cells to eradicate bloodborne bacterial infection caused by Bacillus cereus and methicillin-resistant Staphylococcus aureus [40]. Moreover, they interact with neutrophils to form a neutrophil extracellular trap that sequesters bacteria [41]. However, platelets invariably show diminished function and numbers after severe infection. For example, patients with sepsis often exhibit thrombocytopenia, which is associated with poor prognosis [42-44]. The mitochondrial dysfunction in platelets observed in sepsis and bacterial infection can lead to apoptosis: Bcl-xL—an essential regulator of platelet survival—is upregulated in the platelets of sepsis patients[45, 46]. Autophagy is important for platelet functions, including haemostasis and thrombosis[47]. In our study, UCMSC treatment reversed the decrease in the autophagy marker LC3-II caused by MCAO and E. coli infection.Mitochondria are the main target of the intrinsic apoptosis pathway, and mitochondrial membrane depolarization serves as a marker of apoptosis [48]. ATP provided by mitochondria plays an important role in normal cellular functioning, including the response to physiological stress [49]. Thus, a decrease in ATP levels reflects platelet damage. MMP depolarization is also used as a marker of apoptosis in nucleated cells and anucleate platelets [50].Platelet MMP reflects disease severity in patients with sepsis and correlates with clinical out-

come [51]. In the present study, UCMSC treatment increased the expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL, while restoring MMP and ATP production in platelets. In vitro, UCP MSC and platelets can synergistically inhibit the proliferation of Escherichia coli.So we conclude that UCMSC may play a protecd tive role against post-stroke infection by restoring the count and the function of platelet.

## Conclusions

These results suggest that UCMSC have the ability to modulate the function of $CD8^+$ T cells, NK cells. Our study serves as the basis for future studies and offers new insights into the mechanisms responsible for the beneficial effect of UCMSC transplantation in patients with stroke and post-stroke infection.

## Declarations

**Ethics approval and consent to participate**

All experimental procedures and animal care were performed in accordance with the guidelines and approval of the Animal Ethics Committee of Southern Medical University and were conducted in accordance with the policy of the National Institutes of Health on the care and use of animal

## Data Availability

All the data and informations used and/or analyzed during the current study available from the corresponding author on reasonable request.

## Competing interests

The authors declare that they have no competing interests.

## Author's contributions

JH, ZF, YX and XJ designed the experiments; HT S, FL, and Q OUYANG performed the experiments; ZZ performed data collection and analysis; X Z, YC, YZ and YT wrote the manuscript together; XJ guided this study, revised the manuscript and provided financial support. All authors performed the final approval of the manuscript.

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