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Original Article

The protective effects and underlying mechanisms of Kaempferol on sepsis associated cognitive impairment in rats

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ABSTRACT

Objectives: The neuroprotective effects of Kaempferol (KMF) have been previously reported; however, its possible effects on sepsis-associated encephalopathy remain unclear. This study aimed to investigate the effects and underlying mechanisms of KMF on cognitive impairment in a cecal ligation and puncture (CLP)-induced sepsis model.

Methods: Male Wistar rats were subjected to the CLP model. The animals were divided into four groups: sham, sham + KMF, CLP, and CLP + KMF, and treated with KMF (50 mg/kg, i.p.). Twenty-four hours after CLP, the levels of cytokines, NF-κB, myeloperoxidase (MPO) activity, oxidative damage to lipids and proteins, and antioxidant enzyme activities were evaluated in the hippocampus. Ten days after sepsis induction, behavioral tests were conducted to assess cognitive damage.

Results: KMF reduced TNF-α and IL-1β levels, MPO activity, NF-κB protein levels, and the expression of TLR4 and MYD88 in the hippocampus of septic rats. KMF decreased oxidative stress parameters (MDA and protein carbonyl groups) and increased the activity of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). Additionally, the expression of genes involved in the antioxidant defense system, such as nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1), was upregulated following KMF treatment.

Conclusion: These findings indicate that KMF exerts protective effects on survival rate and cognitive dysfunction after sepsis by inhibiting inflammatory responses and oxidative stress.

Keywords: Kaempferol, Cecal Ligation and Puncture, Sepsis, Inflammation, Cognitive Impairment, Oxidative Stress

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Introduction

Sepsis is a systemic inflammatory response to infection and a major cause of mortality in critical care settings (1). Sepsis-associated encephalopathy (SAE) is a common neurological complication of sepsis, occurring in approximately 70% of septic patients (2). SAE is associated with increased morbidity and mortality worldwide. Clinical manifestations of SAE include acute and long-term cognitive impairments as well as psychological disorders such as anxiety and depression (3). The pathophysiology of SAE is complex and multifactorial, involving several intertwined mechanisms, including vascular damage, endothelial activation, blood-brain barrier breakdown, altered brain signaling, brain inflammation, and apoptosis (4). Inflammatory responses play a central role in the pathophysiology of brain dysfunction in SAE. Elevated pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin (IL)-6, and IL-1β, in the blood and hippocampus contribute to endothelial damage and blood-brain barrier disruption, ultimately leading to neuronal dysfunction and cell death (5). Oxidative damage is another key mechanism involved in SAE. In models of polymicrobial sepsis induced by cecal ligation and perforation (CLP), oxidative damage occurs in the hippocampus, leading to increased production of free radicals (6). At present, therapeutic options for SAE are limited and primarily focus on symptom control. Targeting mechanistic pathways underlying SAE may provide new treatment approaches for this disorder. Therefore, strategies aimed at reducing both inflammation and oxidative stress could hold potential therapeutic value for SAE treatment.

Kaempferol (KMF) is a flavonoid antioxidant found in fruits and vegetables. Consumption of KMF has been associated with a wide range of health benefits, including neuroprotection, cardioprotection, weight loss, chemopreventive properties, anti-inflammatory activity, reduced blood pressure, decreased diet-induced insulin resistance, anxiolytic effects, and hypoglycemic and hypolipidemic impacts (7). In vitro and in vivo studies provide evidence that KMF supplementation may protect against neurological degeneration and oxidative stress-related diseases in the brain. One study reported that KMF improves cognitive function through the inhibition of acetylcholinesterase activity (8). Another study suggested that KMF significantly attenuates cognitive damage in mice through its antioxidant action (9). Moreover, the preventive effect of KMF on cognitive deficits via regulation of oxidative stress and neuroinflammation in an ovariectomized rat model of sporadic dementia has been documented (10). Lopez-Sanchez et al. demonstrated that KMF administration prevented the enhanced production of amyloid β peptides in the striatum and hippocampus of mice (11). Additionally, research suggests that KMF protects against cerebral ischemia/reperfusion injury in rats by attenuating neuroinflammation and blood-brain barrier dysfunction, thereby improving neurological deficits (12). Furthermore, KMF has been shown to exert a neuroprotective effect by reducing neuropathic pain through the toll-like receptor 4 (TLR4) / nuclear factor κB (NF-κB) signaling pathway (13). Despite substantial research highlighting the neuroprotective effects of KMF, data regarding its impact on cognitive impairment associated with sepsis remain limited. Therefore, the present study aimed to evaluate the effects of KMF on survival rate and cognitive dysfunction following sepsis induction by CLP. Additionally, we examined the possible underlying mechanisms of KMF's neuroprotective effects, focusing on inflammatory responses and oxidative stress status in the hippocampus.

Materials and Methods

Animals

Male Wistar rats (3–4 months, 250–300 g) were housed three per cage, with food and water available ad libitum, and maintained on a 12-hour light/dark cycle. The animals were fasted overnight before surgery. The study was conducted in accordance with the procedures established by the Institutional Animal Ethics Committee.

Cecal ligation and perforation (CLP) model

The animals were subjected to sepsis induction by CLP, as previously described (14). Briefly, the rats were anesthetized via intraperitoneal injection of a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). A 3-cm incision was made in the lower abdomen, exposing the cecum, which was ligated below the ileocecal valve and punctured once with a sterile 14-gauge needle. Subsequently, the abdominal cavity was closed using aseptic surgical sutures, and the rats received a subcutaneous injection of saline solution (3 mL/100 g body weight). For the sham-operated groups, laparotomy was performed, and the cecum was manipulated but not ligated or perforated.

Treatments and samples

Animals were randomly divided into four groups: sham+ saline; sham +KMF, CLP + saline, and CLP+ KMF.For inflammatory and oxidative stress analyses, 7 animals per sham group and 12 animals per CLP group were used. For behavioral tests, 12 animals per sham group and 18 animals per CLP group were included. For treatment, KMF was administered intraperitoneally (i.p.) at a dose of 50 mg/kg (Sigma-Aldrich, purity 95%; St. Louis, MO, USA) (15). Rats in the sham and CLP groups received an equal volume of normal saline. For inflammatory and oxidative stress assays, two doses of KMF were administered immediately after surgery and 12 hours post-procedure. In behavioral analyses, rats

received a daily dose of KMF or saline for 10 days. At 24 hours or 10 days post-operation, the rats were subjected to painless euthanasia using thiopental overdose (0.5 g/kg) followed by decapitation. The hippocampus was rapidly excised and stored at −80°C for subsequent analysis.

Determination of oxidative stress parameters

The level of lipid peroxidation in the hippocampus was measured based on malondialdehyde (MDA) quantification via the thiobarbituric acid (TBA) reaction. Results were expressed as MDA equivalents (nmol/mg protein). Protein carbonyl levels, indicative of protein oxidation, were measured based on their reaction with dinitrophenylhydrazine. Briefly, proteins were precipitated with 20% trichloroacetic acid, dissolved in dinitrophenylhydrazine, and absorbance was recorded at 370 nm. Results were expressed as protein carbonyl content per milligram of protein.

The activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) (Nanjing Jiancheng Bioengineering Institute, China) were assessed following kit specifications. Results were presented as enzyme activity units per milligram of protein.

Determination of inflammatory parameters

TNF-α and IL-1β concentrations in the hippocampus were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) following the manufacturer's instructions. Data were expressed as picograms per milliliter (pg/mL). Additionally, myeloperoxidase (MPO) activity, an indicator of neutrophil migration, was measured using H_2O_2, hexadecyltrimethylammonium bromide (HTAB), and 3,3',5,5' tetramethylbenzidine (TMB) (16). Results were expressed as mU per milligram of protein.

Western blot analysis

Cytoplasmic and nuclear proteins from the hippocampus tissues were isolated using a nuclear and cytoplasmic protein extraction kit (Beyotime Institute of Biotechnology, Haimen, China). Protein concentrations were determined by the bicinchoninic acid assay (Wellbio, China). The equal amounts of protein samples were separated by SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). After blocking the membranes with 5% skim milk TSB buffer for 2 h, we incubated them with primary antibodies: Histone1(Abcam, USA), β-actin (Sigma, USA), IκBα (Cell Signaling Technology, Boston, USA), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB((Cell Signaling Technology, USA). After being incubated with the corresponding secondary antibodies at room temperature for 2 h, the blots were visualized with an enhanced chemiluminescence (ECL) detection system (GE, Healthcare Life Sciences), and the intensity of each band was quantified with densitometric analyses (Bio-Rad Laboratories).

Quantitative PCR analysis

Total RNA was extracted using the RNApure Tissue & Cell Kit, following the manufacturer's instructions (CWBIO, China). A cDNA synthesis kit was used to reverse transcribe RNA into first-strand cDNA. TransStart Green q-PCR SuperMix (TransGen Biotech, China) was used to perform real-time PCR. To normalize expression data, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference gene. Relative gene expression was calculated using the $2^{-\Delta Ct}$ method.

Behavior tests

Open field test

The open field test was conducted in a wooden box (50×60×60 cm) divided into 16 regular square areas. Each rat was placed at the center of the box at the beginning of the session and allowed to explore the arena for 5 minutes. A decrease in the number of crossings and rearings between the training and test sessions was used as a measure of habituation retention (14).

Object recognition test

The object recognition test was conducted in the open field. Briefly, all animals underwent a habituation session, where they freely explored the open field for 5 minutes over two consecutive days. No objects were placed in the box during the habituation period. For training, two identical objects (A1 and A2) were placed in the apparatus, and the rats were allowed to freely explore them for 5 minutes. The short-term recognition memory test was performed 1.5 hours after training. The rats explored the open field for 5 minutes in the presence of one familiar (A) and one novel (B) object. The long-term recognition memory test was performed 24 hours after the training session. Exploration was defined as sniffing or touching the objects with the nose and/or forepaws. A recognition index calculated for each animal is reported as the ratio TB/(TA+TB), as TA = time spent exploring the familiar object A and TB = time spent exploring the novel object B (14).

Statistical analysis

All statistical analyses were performed using SPSS software 23.0 (IBM, Chicago, IL, USA). Data were presented as mean ± standard deviation (S.D.). Comparison of multiple groups was performed using one-way analysis of variance (ANOVA). If ANOVA indicated a significant difference, the Tukey post hoc test was applied. For behavioral tests, data were expressed as median ± interquartile range, and differences among groups were determined using the Mann-Whitney

and Wilcoxon tests. In all comparisons, statistical significance was set at $p < 0.05$.

Results

Kaempferol increased the survival rate of septic rats

First, we evaluated the effect of KMF on the survival rate in the septic model. The 7-day survival rate in the sham group was 100%. However, 7 days after CLP surgery, the survival rate decreased dramatically (33%) compared with the sham group ($P < 0.05$). Administration of KMF led to a marked increase in the survival rate (63%) in the CLP + KMF group compared to the CLP group ($P < 0.05$) (Fig. 1).

Kaempferol reduced the levels of pro-inflammatory cytokines in the hippocampus of CLP-induced sepsis rats

We investigated the impact of KMF on inflammation in the hippocampus of septic mice 24 hours after CLP surgery. In the CLP group, levels of pro-inflammatory cytokines, including TNF-α and IL-1β, were markedly increased compared to the sham group ($P < 0.01$) (Fig. 2A-B). Kaempferol treatment effectively reduced the levels of these cytokines in the hippocampus of CLP septic mice ($P < 0.01$). We also assessed MPO activity, a marker of neutrophil infiltration. Our results revealed that sepsis led to a higher MPO activity compared to the sham group, whereas KMF treatment significantly decreased MPO activity ($P < 0.01$) (Fig. 2C). To further explore KMF's anti-inflammatory effects, we examined its impact on the NF-κB signaling pathway using western blot analysis. The CLP group exhibited elevated nuclear NF-κB levels and downregulated cytoplasmic IκBα levels compared to the sham group ($P < 0.01$). However, KMF intervention significantly suppressed nuclear NF-κB expression while enhancing cytoplasmic IκBα levels in the CLP group ($P < 0.01$) (Fig. 2D-E). Additionally, we evaluated the expression of TLR4 and myeloid differentiation factor 88 (MyD88), two key molecules involved in lipopolysaccharide (LPS) recognition. Compared with the sham group, the CLP group exhibited significantly elevated TLR4 and MyD88 mRNA expression ($P < 0.01$). However, KMF treatment markedly inhibited CLP-induced TLR4 and MyD88 expression in the hippocampus ($P < 0.01$) (Fig. 2F-G).

Kaempferol attenuated oxidative stress in the hippocampus of CLP-induced sepsis rats

As demonstrated in Figure 3, the activities of antioxidant enzymes (SOD, CAT, and GPx) and the levels of oxidative products (MDA and protein carbonyl groups) were assessed in the hippocampus of each experimental group 24 hours after surgery. The levels of MDA and protein carbonyl groups were significantly increased in the CLP group compared to the sham group ($P < 0.01$). However, KMF treatment markedly reduced CLP-induced MDA and protein carbonyl group levels (Fig. 3A, D). Conversely, SOD, CAT, and GPx activities were decreased following CLP, whereas KMF treatment significantly enhanced the activity of these enzymes (Fig. 3B, C, E). We also measured the expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and its target gene, heme oxygenase-1 (HO-1). Importantly, the expression levels of both genes were significantly upregulated following KMF treatment (Fig. 3F-G).

Kaempferol prevented memory impairment in CLP-induced sepsis rats

In the open field test, the number of crossings and rearings was recorded to evaluate motor and exploratory activity in the animals. Significant reductions were observed in crossings and rearings in the sham and sham + KMF groups during the test session. In contrast, the septic CLP group showed no differences between

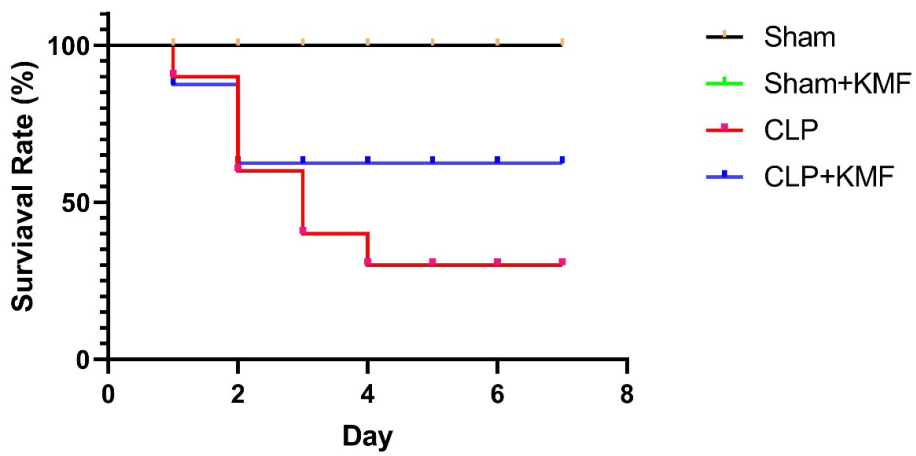

Figure 1. Comparison of the survival rate of the rats in different groups 7 days after surgery. Seven days after surgery, the survival rates of the Sham, Sham + KMF, CLP, and CLP+KMF groups were 100%, 100%, 33, and 63%, respectively. The survival rates of the CLP+KMF group was significantly higher than those of the CLP group. * $P < 0.01$ compared with the Sham group; # $P < 0.01$ compared with the CLP group.

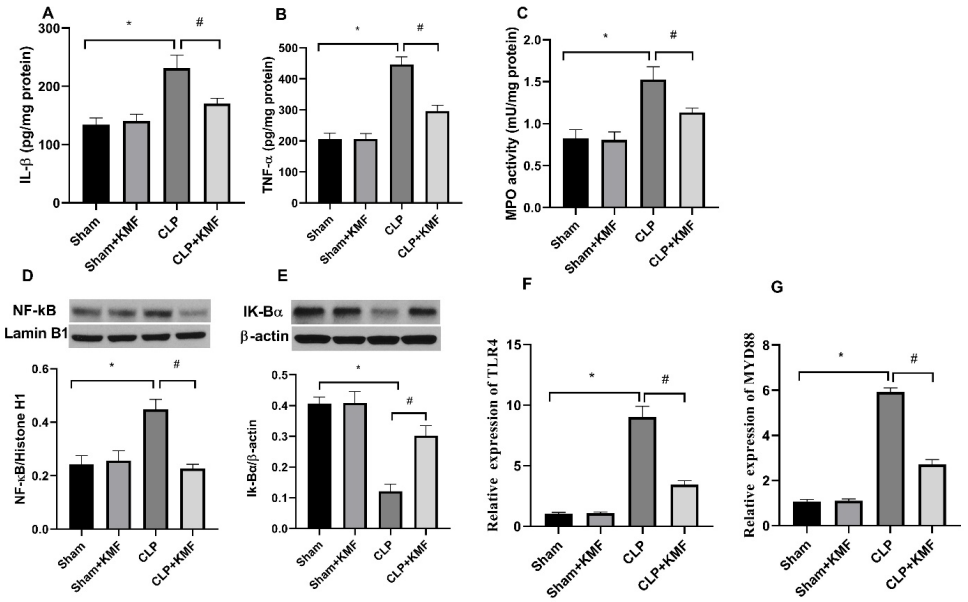

Figure 2. Effects of KMF on inflammatory markers in the hippocampus of different groups. Hippocampi were homogenized and TNF-α (A), IL-1β (B) levels, and MPO activity (C) were measured using commercial kits. The nuclear NF-κB (D) and the cytoplasmic IκBα (E) were determined using Western blot analysis. The expression of TLR4 (F) and MYD88 (G) was measured using real time PCR. Data are represented as mean± SD. * $P < 0.01$ compared with the Sham group; # $P < 0.01$ compared with the CLP group.

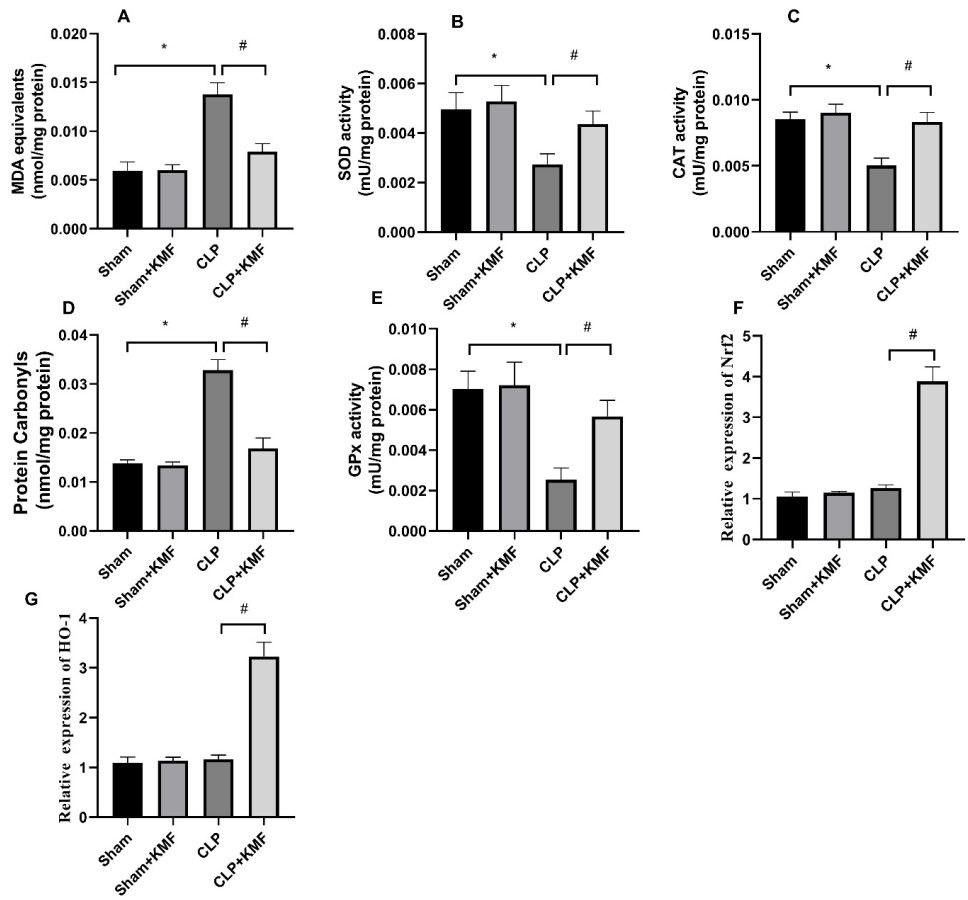

Figure 3. Effects of treatment with KMF on oxidative stress markers. The level of MDA (A), the activities of SOD (B), CAT (C), the levels of protein carbonyls groups (D) and the activity of GPx (E) were measured in the hippocampus of different groups. The expression of Nrf2 (F) and HO-1 (G) was measured using real time PCR. Data are represented as mean± SD. * $P < 0.01$ compared with the Sham group; # $P < 0.01$ compared with the CLP group.

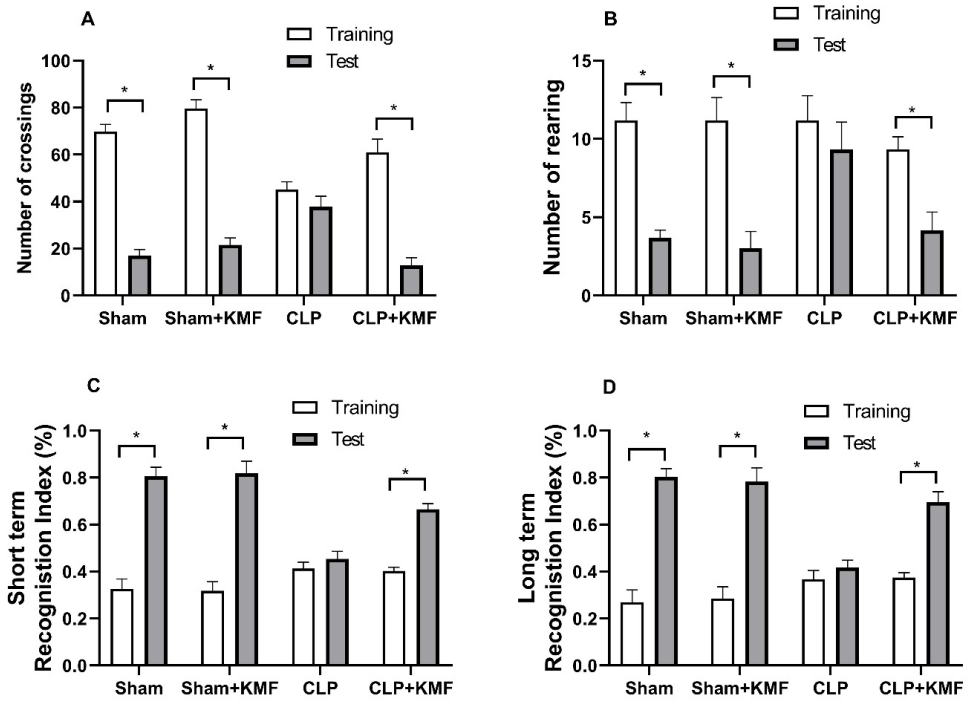

Figure 4. Effect of KMF on the memory function in the rats with CLP-induced sepsis. A) Effect of KMF on the number of crossings (A) and rearings (B) on the open field test. Short-term (C) and long-term (D) object recognition index of rats subjected to sepsis and treated with KMF. Data are presented as median ± interquartile rage, analyzed by Mann-Whitney and Wilcoxon test. *p<0.01 vs. test session.

the training and test sessions in crossing and rearing parameters, indicating impairment in spatial habituation retention. However, CLP animals treated with KMF exhibited behavior similar to the sham animals, suggesting no memory deficit (Fig. 4A-B). For the object recognition test, animals subjected to sepsis displayed memory impairment in recognizing a new object in both short-term (Fig. 4C) and long-term evaluations (Fig. 4D). KMF treatment significantly prevented memory deficits in both short-term and long-term evaluations, indicating an improvement in cognitive function.

Discussion

Previous studies have demonstrated that KMF can attenuate inflammation and oxidative stress in various pathological conditions; however, its effects on inflammatory responses and oxidative stress status during sepsis have not been investigated. To the best of our knowledge, this is the first study to explore the protective effects of KMF in CLP-induced sepsis in rats.

Brain dysfunction in sepsis is associated with the generation of pro-inflammatory cytokines, leading to cognitive impairment. Microglia activation and the subsequent release of pro-inflammatory cytokines, including TNF-α and IL-1β, are hypothesized to play key roles in the development of SAE (17). Consistent with previous findings, we observed an increase in TNF-α and IL-1β levels in the hippocampus of septic rats (18, 19). It is noteworthy that while the anti-inflammatory effects of KMF have been reported in various cells and tissues, its impact on the brain in septic models has not been previously explored (20).

The activation of NF-κB signaling plays a central role in the inflammatory response during sepsis. NF-κB activity is markedly enhanced in multiple organs of both animal models and human subjects with sepsis (21). Increased NF-κB activity has been linked to higher mortality rates and worse clinical outcomes (22). Under unstimulated conditions, NF-κB protein is predominantly localized in the cytoplasm, where it is bound to a family of inhibitory proteins, including IκBα (23). Upon stimulation, phosphorylation of IκBα by IκB kinases leads to ubiquitination and proteasomal degradation of IκBα, resulting in the release of NF-κB, which subsequently translocates to the nucleus and binds to promoter elements, initiating gene expression (24). In the current study, we observed localization of IκBα in the cytoplasm and NF-κB in the nucleus, indicating NF-κB pathway activation in the hippocampus of CLP-induced septic rats. Additionally, we found that KMF treatment effectively disrupted NF-κB activation by inhibiting IκBα degradation and preventing NF-κB nuclear translocation. This finding aligns with a previous study, which demonstrated that KMF could inhibit NF-κB signaling in spinal cord injury rats (12).

Toll-like receptors (TLRs) belong to the

transmembrane protein family and play a critical role in the regulation of inflammatory and innate immune responses. Among them, TLR4 specifically recognizes endogenous molecules released from damaged or ischemic tissues, termed danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) (25). The association of TLR4 with its adaptor protein, MYD88, activates the NF-κB pathway, leading to the production of pro-inflammatory mediators (26). In the present study, we observed an increase in the expression of TLR4 and MYD88 in septic rats, whereas KMF treatment effectively reduced the expression of these molecules in the hippocampus. Similarly, Yang et al. (27) demonstrated that KMF attenuates LPS-induced striatum injury in mice via downregulation of the TLR4 pathway. Taken together, our findings suggest that KMF effectively decreases the production of IL-1β and TNF-α in the hippocampus of CLP-induced septic rats. Furthermore, KMF inhibits TLR4 and MyD88 expression, along with NF-κB activation, indicating that its anti-inflammatory effects in sepsis may be mediated through inhibition of the TLR4/MyD88/NF-κB signaling pathway.

Oxidative stress is a pivotal factor in triggering brain injuries associated with sepsis. The overproduction of reactive oxygen species (ROS) leads to lipid peroxidation, protein oxidation, and subsequent damage to cell membranes, mitochondria, and neuronal apoptosis (28). Alterations in oxidative stress markers, such as elevated MDA levels, increased protein carbonyl groups, and reduced antioxidant enzyme activity (SOD and CAT), have been observed in the brains of septic animals (14, 18). In our study, KMF treatment was associated with enhanced activities of antioxidant enzymes (SOD, CAT, and GPx) and decreased levels of oxidative products (MDA and protein carbonyls) in the hippocampus of septic rats. At the molecular level, KMF significantly potentiated Nrf2 signaling by increasing mRNA expression of Nrf2 and its downstream target gene, HO-1. Therefore, attenuation of oxidative stress in the hippocampus may represent a key mechanism underlying the preventive effects of KMF in sepsis-induced brain injury. The role of KMF in controlling oxidative stress has previously been demonstrated (10).

In our study, we employed habituation to an open field and novel object recognition tasks to investigate memory impairment. Previous studies have demonstrated that sepsis-induced oxidative damage and inflammatory responses occur in the hippocampus of rodents after CLP, and that antioxidants capable of preventing oxidative changes in the hippocampus can attenuate learning and memory impairment following CLP (29). Our findings indicate that KMF treatment effectively mitigates cognitive dysfunction induced by CLP in rats. Taken together, these data suggest that the mechanism underlying KMF's ability to reverse memory decline in sepsis is likely linked to the inhibition of oxidative damage and inflammatory responses.

Conclusion

In summary, our findings demonstrate that KMF treatment effectively reduces oxidative damage in lipids and proteins, as well as attenuates inflammatory responses in the hippocampus of CLP-induced septic rats, ultimately leading to improved cognitive function. This study provides valuable evidence supporting the potential clinical application of KMF for the treatment of sepsis-induced brain injury.

Conflict of interests

The authors declare no conflict of interest.

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There is no funding in this study.

Data Availability Statements

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

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