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RESEARCH ARTICLE

Protective Impacts and Involved Mechanisms of Chlorogenic Acid on Sepsis-Associated Cognitive Deficits in Rats

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A B S T R A C T

The neuroprotective attributes of chlorogenic acid (CGA) have been delineated in prior literature, yet its precise role in addressing encephalopathy associated with sepsis remains to be elucidated. The primary objective of this study was to delineate the neurocognitive effects of CGA and to ascertain the underlying mechanisms of its action in a caecal ligation and puncture (CLP)-induced model of sepsis. Male Sprague-Dawley rats were subjected to sepsis via the CLP procedure and subsequently segregated into distinct cohorts: (1) CLP + CGA; (2) CLP + saline; (3) sham + CGA; and (4) sham + saline. The experimental paradigm entailed the intraperitoneal administration of 30 mg/kg CGA. In the novel object recognition assay, septic rats manifested discernible deficits in memory retention, as evidenced by their diminished ability to recognize novel objects during both acute and protracted assessments. Contrastingly, CGA administration efficaciously attenuated these cognitive shortcomings, signifying an enhancement in mnemonic function. Concurrently, in the open field assessment, CGA-treated rats mirrored the behavioural patterns exhibited by sham-operated counterparts, alluding to the preservation and potential augmentation of cognitive faculties. Collectively, these observations underscore the putative therapeutic efficacy of CGA in ameliorating cognitive dysfunctions and mnemonic deficits engendered by sepsis.

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INTRODUCTION

Sepsis consistently ranks as a predominant cause of mortality in critical care units, often originating from a systemic inflammatory response instigated by infectious agents (Caldas *et al*., 2022). This pervasive medical challenge, while affecting multiple organ systems, disproportionately impacts the brain, leading to the often-devastating sepsis-associated encephalopathy (SAE) (Xiong *et al*., 2023). Alarmingly, SAE is reported to manifest in approximately 70% of individuals diagnosed with sepsis, thereby accentuating the already significant global morbidity and mortality rates associated with this condition (Chung *et al*., 2020; Li *et al*., 2020). From a clinical perspective, the repercussions of SAE extend beyond immediate cognitive deficits. Patients frequently grapple with long-term cognitive challenges and may also develop a range of psychological disorders, including but not limited to anxiety and depression. Such multifaceted complications further hinder the recuperation process, complicating the clinical management and rehabilitation of affected individuals (Czempik *et al*., 2020)

Venturing into the underlying mechanisms that give rise to SAE, its pathophysiology is intricate and multifactorial. While the complete picture remains partially obscured, current understanding points towards a convergence of factors detrimental to neuronal health. These encompass neurotoxicity, apoptosis, pervasive neuroinflammation, oxidative stress, disruptions in the integrity of the blood-brain barrier, and mitochondrial anomalies, all of which collectively pave the way for SAE's onset (Nwafor *et al*., 2019; Zhang *et al*., 2021). Amplifying this neurotoxic environment is the surge in pro-inflammatory cytokines, notably interleukin-1β,

interleukin-6, and tumour necrosis factor α (TNF-α). These cytokines, beyond being elevated in systemic circulation, also permeate the brain, particularly the hippocampus, exacerbating the existing neuroinflammatory state (López-Taboada *et al*., 2022). Amidst the onslaught of sepsis, the hippocampus-a neural hub central to memory and cognitive processes-finds itself susceptible to oxidative damage. This vulnerability arises due to an escalated production of free radicals, further compounding neuronal damage and dysfunction (Gould *et al*., 2023).

Emerging from the backdrop of potential therapeutic agents is chlorogenic acid (CGA). Derived from a myriad of commonplace dietary sources-including tomatoes, carrots, coffee, grapes, and apples-CGA has carved a niche for itself in the realm of health-promoting compounds (Cheng *et al*., 2022). Its multifaceted benefits span across a range of physiological processes, from imparting neuroprotective and cardioprotective effects to modulating inflammation and mitigating oxidative stress. Additionally, CGA has showcased promise in ameliorating conditions like insulin resistance, hypertension, weight anomalies, and even psychological disorders such as anxiety, making it a compound of considerable interest in the broader medical community (Gao *et al*., 2020; Dada *et al*., 2021). A plethora of clinical and experimental investigations have underscored CGA's potency in staving off neurodegeneration and other neuronal pathologies primarily driven by oxidative stress (Patwa *et al*., 2022). Notably, studies have illuminated CGA's capability to counteract hydrogen peroxide-induced neuronal apoptosis and to reverse memory impairments instigated by compounds like scopolamine (Ju *et al*., 2019; Liu *et al*., 2020). Adding to its burgeoning profile, there's compelling evidence pinpointing CGA's efficacy in attenuating Aβ overexpression in neuronal cells and in enhancing cognitive processes in animal models (Pan *et al*., 2022).

Yet, despite the accumulating evidence in favour of CGA's neuroprotective prowess, a significant knowledge gap persists regarding its effectiveness in ameliorating cognitive deficits specifically associated with sepsis. Our research endeavour aims to bridge this gap by delineating CGA's potential impact on survival outcomes and cognitive integrity amidst the multifaceted challenges of sepsis. Through a rigorous exploration of the complex mechanisms underpinning CGA's neuroprotective actions, our study seeks to discern its capacity to modulate anti-inflammatory pathways and to temper oxidative stress, particularly within the critical structure of the hippocampus. As this research narrative evolves, CGA emerges not merely as a molecule of interest, but as a beacon of hope in the quest to better understand and manage the cognitive repercussions of Sepsis-Associated Encephalopathy (SAE).

MATERIALS AND METHODS

Materials: Our study used the following reagents and chemicals; N9 cells (Cell Bank of the Chinese Academy), CLP (Sigma−Aldrich, USA), Chlorogenic acid (Shanghai, China). The following antibodies were used, anti-iNOS, anti-IL-10, anti-CD86, anti-MMP9, anti-Arg-1, anti-CD206, and anti-CXCL12 all from Fisher Scientific (USA). Cleaved caspase-3, p-Akt1, Akt1, and p-ERK1/2 from Wuhan (China).

Animals: Male Sprague-Dawley rats, aged 10-12 weeks, were housed in plastic cages with dimensions of 16" ×10" ×9" (three rats per cage) within a well-ventilated room set at a temperature of 23±2°C, operating on a 12-hour light-dark cycle. A standard diet and water were made freely available to all rats throughout the experimental period. The study was conducted according to the procedures laid down by Animal Ethics in China. 96 rats were randomly included in one of the following groups each consisting of 24 rats: (1) CLP + CGA; (2) CLP + saline;(3) sham + CGA and (4) sham + saline. For inflammatory and oxidative stress analyses, there were 14 rats used for each sham group and 24 rats for the CLP groups. CGA was administered in two doses, the first one right after the surgery and the second one 12 hours succeeding the procedure. In behaviour analyses, the rats were given either CGA or saline on a daily basis for10 days.

These groupings and protocols were determined from previous studies, from the 7th day, the doses of CGA were subsequently reduced in steps of 10 mg/kg/day for another 7 days. Solvent control was achieved using DMSO at 5% intraperitoneally in the sham and CGA groups. In behaviour tests, there were 24 rats used for each sham group and 36 rats included in the CLP groups. For the treatment, CGA (30 mg/kg) was injected intraperitoneally (Sigma-Aldrich, purity 495%) [19] daily for 10 days. These tests were conducted between day 7 and 10 after euthanizing the rats with sodium pentobarbital. Rats were murdered painlessly by injecting them (with thiopental (0.5 g/Kg) and decapitation 24 hours or 10 days following the surgeries. The hippocampus of rats was rapidly excised and stored (-80ºC) for further examinations.

Assessment of Neurobehavior: The scores on neurobehaviour were determined from four factors depending on the auricular reflex, flip-right reflex, corneal reflex, caudal flexion and escape response. The scores were tabulated from "0", implying no reflex, "1", implying a reflex time greater than 1s, and a score of "2" showing a reflex time of exactly 1 second. The neurobehaviour scores were inversely proportional to the destruction of the nervous system and the brain. These scores were evaluated using three independent single blind research personnel.

Open Field Experiment and Water Maze Test: In the open field experiment, all the rats were acclimatized to the testing environment for three hours. A white experimental chamber (130 cm×130 cm×140 cm) was provided for free movement. We adjusted various parameters and recorded date and rat count, placed them in the central area, and monitored their 15-minute movement using behaviour analysis software. Furthermore, total distance moved, central area entries, time percentage in the central area, and speed-based activity were recorded.

In the water maze test, a circular platform was introduced into a basin with a diameter of 80cm, and the basin was filled with tap water at a temperature of 22°C

(water surface-maintained 5cm above the platform). Before the administration of CGA, four groups of rats underwent two days of training sessions aimed at locating a concealed platform within 45 seconds. Rats unable to find the platform within the time limit were assisted to it for a duration of 8 seconds. On the fifth day following CGA injection, the spatial navigation test was conducted, capturing the escape latency. The subsequent day, the platform was removed, allowing the rats to explore freely for 45 seconds. Metrics including movement trajectory, mean swimming speed, total distance swum, and the proportion of time spent in the target quadrant were documented.

Immunohistochemistry and Fluorescence: All the rats were euthanized using a perfused saline and 6% paraformaldehyde through the heart. Brain tissues were removed and fixed in 6% paraformaldehyde overnight. Following this, the tissue underwent dehydration using 99% ethanol before being encased in paraffin to create brain sections that were 2µm in thickness. Subsequently, the paraffin-encased sections underwent a water-based dewaxing process, followed by a sequential hematoxylin and eosin staining procedure. The stained sections were dehydrated, sealed, and subjected to microscopic examination to assess potential neuronal impairment within the hippocampal region of the murine brain.

The section was then dewaxed to retrieve antigens before a uniform covering using 5% BSA preceded by 15-minute blocking at room temperature. Cleaved caspase-3 was the primary target antibody applied dropwise and incubated under humid conditions at 4°C. Secondary antibodies were introduced and allowed to incubate at room temperature with DAB used for developing the colours from brown to yellow solutions. A restaining process involving hematoxylin, lasting approximately 3 minutes, was conducted, followed by washing.

Paraffin-embedded sections were dewaxed, followed by antigen retrieval and room temperature blocking with 5% BSA. Primary antibodies (IBA-1 and CD86/CD206) were added dropwise and incubated overnight at 4°C. Corresponding secondary antibody was applied at room temperature incubation, followed by DAPI staining for nuclei. After washing, sections were sealed. Fluorescence microscope was used to image microglia polarization in hippocampal region of murine brain.

Cell culture, Assays and Western blotting Analyses: The N9 cells were cultured in MEM (15% serum, 1% penicillin and 1% streptomycin). The conditions were 37°C and 5% carbon dioxide. Western blotting was performed by electrophoresing the protein samples in 15% SDS polyacrylamide gels before transforming into PVDF membranes and blocked with 5% skimmed milk for 120 mins. Chemiluminescence was used to enhance the PVDF membranes. Flow cytometry assay was performed by transferring the cells to centrifuge tubes and washing with PBS before blocking in 5% BSA. The primary and secondary antibodies were then added at room temperature for 120 mins. In wound healing assay, the N9 cells were inoculated in six plates and cultured to achieve cell density of 90%. 2 days after scratching, the rates of cell migration were measured using a microscope. Transwell assay involved filling the upper chamber with MEM (5% serum) and lower chamber (1% serum). Unperforated cells were removed using a cotton swab and the perforated cells were observed and counted using a light microscope.

Statistical analysis: The statistical analysis was performed using GraphPad Prism software (USA, version 9.52.1). The reported data were presented as means with corresponding standard deviations (SD). Multiple group comparisons utilized one-way analysis of variance (ANOVA). In cases where ANOVA tests revealed significant differences, the Tukey post hoc test was conducted for pairwise comparisons between groups. Behavioural test results were presented as median ± interquartile range (IQR), and comparisons were assessed using Mann-Whitney and Wilcoxon tests. Statistical significance was set at $P<0.05$.

RESULTS

Chlorogenic acid improved the rate of survival in septic rats: According to Fig. 1, rats treated with chlorogenic acid (CGA) during sepsis (CLP + CGA) showed a significantly higher survival rate of 75% compared to septic rats treated with saline (CLP + Saline) with a survival rate of 45%. CGA did not significantly affect survival in rats without sepsis (Sham + CGA, 90%; Sham + Saline, 95%). This suggests that CGA might have a protective effect specifically in sepsis conditions, enhancing survival rates (Fig. 1).

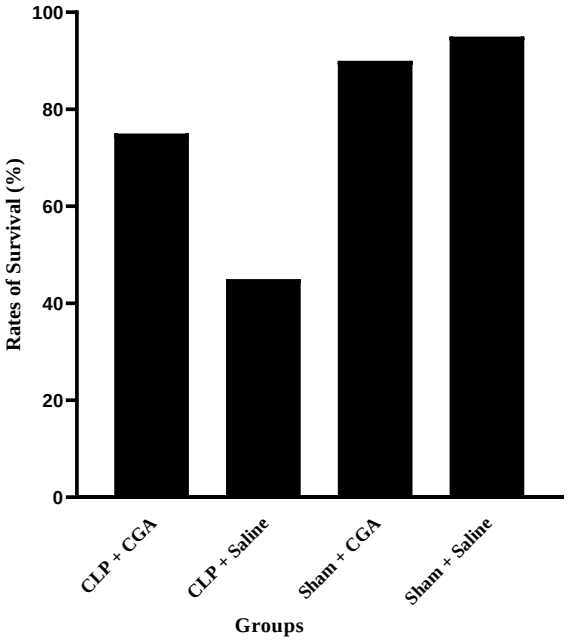

Fig. 1: Rates of Survival after administration of Chlorogenic acid in experimental groups. CGA: Chlorogenic acid; CLP: Caecal ligation and puncture.

Chlorogenic acid reduced hippocampus pro-inflammatory cytokines in CLP-induced sepsis rats: According to Fig. 2, the levels of IL-1β and TNF-α in the hippocampus were significantly reduced in septic rats treated with chlorogenic acid (CLP + CGA) compared to septic rats treated with saline (CLP + Saline), as indicated by t-tests ($P<0.05$). This suggests that chlorogenic acid

administration effectively lowered pro-inflammatory cytokine levels in the hippocampus of CLP-induced sepsis rats.

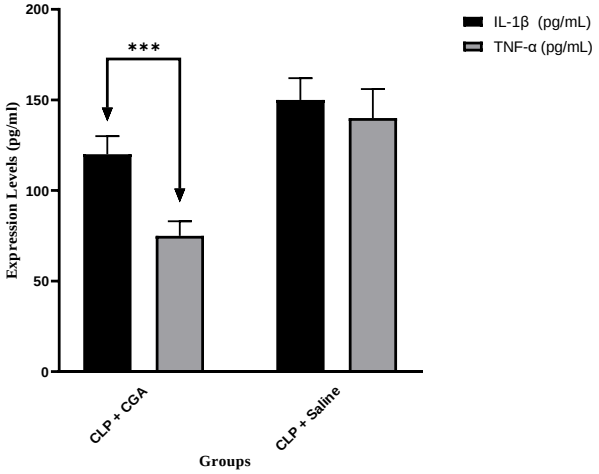

Fig. 2: Expression Levels of IL-1β and TNF-α in the hippocampus. CGA: Chlorogenic acid; CLP: Caecal ligation and puncture.

Chlorogenic acid attenuated oxidative stress in the hippocampus of septic rats: The levels of oxidative stress markers were assessed within 48 hours. According to Fig. 3, the levels of protein carbonyls were significantly reduced in septic rats treated with chlorogenic acid (CLP + CGA) compared to septic rats treated with saline (CLP + Saline) as indicated by t-test ($P<0.05$). Additionally, protein carbonyl levels in the sham-operated groups were not significantly affected by CGA. The MDA levels were significantly lower in both septic and non-septic rats treated with chlorogenic acid (CLP + CGA and Sham + CGA) compared to their respective saline-treated groups (CLP + Saline and Sham + Saline) according to t-tests ($P<0.05$).

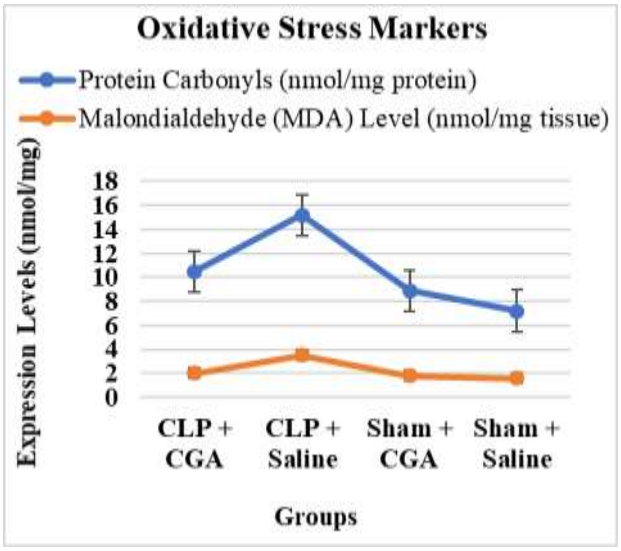

Fig. 3: Expression levels of oxidative stress markers in the experimental groups. CGA: Chlorogenic acid; CLP: Caecal ligation and puncture.

Chlorogenic acid prevented memory deficit in septic rats: In the open field test, both the Sham + CGA and Sham + Saline groups displayed significant reductions in crossings and rearing during test sessions ($P<0.05$), indicating spatial habituation. The CLP + Saline group did not show notable differences in these parameters between test and training sessions, suggesting retention impairments. Conversely, the CLP + CGA group, treated with chlorogenic acid, exhibited behaviours similar to Sham rats ($P<0.05$), suggesting memory preservation and potential cognitive improvement (Fig. 4).

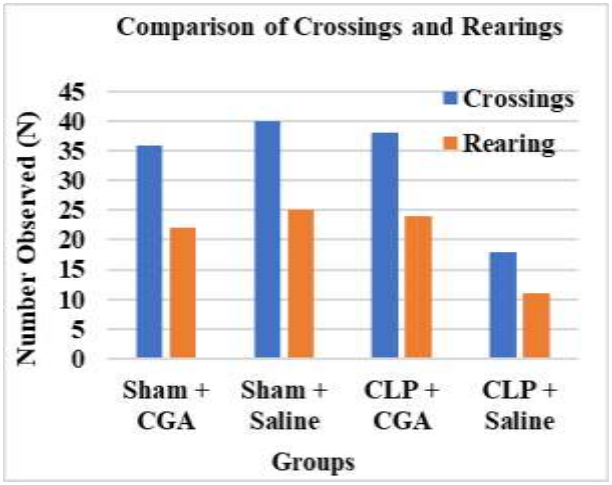

Fig. 4: Comparison of crossings and earrings across experimental groups. CGA: Chlorogenic acid; CLP: Caecal ligation and puncture.

In the object recognition test, sepsis-induced rats exhibited impaired memory for identifying a novel object in both short-term and long-term evaluations. The CLP + CGA group, treated with chlorogenic acid, showed significantly improved memory performance in both short-term and long-term assessments ($P<0.05$), suggesting CGA's effectiveness in mitigating memory impairment and enhancing memory function in septic rats. The CLP + Saline group did not exhibit significant improvement in memory performance.

DISCUSSION

In our recent research, we sought to investigate the possible protective effects of CGA on rats subjected to sepsis caused by CLP. The cognitive impairments seen during sepsis are believed to be due to the emission of pro-inflammatory agents that influence brain activity (Cheng *et al*., 2022). Activation of microglia and subsequent secretion of inflammatory factors, including IL-1β and TNF-α, have been implicated in the development of sepsis-associated encephalopathy (SAE) (Moraes *et al*., 2021; Gasmi *et al*., 2022). CGA is documented to exhibit robust antioxidative capabilities. The phenomenon of oxidative stress, defined by a disparity between deleterious reactive oxygen species (ROS) and the body's antioxidative defense systems, is central to inflammatory processes. In alignment with previous literature, our research identified a pronounced elevation in hippocampal concentrations of TNF-α and IL-1β in rats afflicted by sepsis. It is worth noting that, despite the recognized anti-inflammatory properties of CGA in diverse tissues and cellular contexts, its role within the cerebral milieu of septic models remains largely uncharted (Lv *et al*., 2021).

Activation of NF-kB is fundamental in instigating inflammatory reactions during sepsis. Augmented NF-κB signalling has been documented in both septic human subjects and experimental animal models across an array of organs, and is associated with increased mortality and suboptimal clinical prognoses (Tang *et al*., 2022; Kracht *et al*., 2020). Under unstimulated conditions, NF-kB localizes primarily in the cytoplasm, bound to inhibitory proteins like Ik-Bα (Su *et al*., 2023). Upon activation, IkB kinases instigate the phosphorylation of Ik-Bα, culminating in its ubiquitination followed by proteasomal degradation. Subsequently, NF-kB is liberated, facilitating its translocation to the nucleus and subsequent binding to promoter sequences, thereby driving the initiation of gene transcription (Jain *et al*., 2023). CGA affects important pathways related to inflammation, especially the NF-kB pathway. NF-kB controls the creation of many inflammatory genes. When CGA interferes with this pathway, it reduces the production of inflammatory substances like IL-1β and TNF-α.

In our study, we found evidence of the NF-kB pathway being active in the brains of septic rats. CGA was effective in stopping this activity. It did this by preventing the breakdown of a key molecule (IκBα) and stopping NF-kB from moving to the nucleus where it activates genes. Essentially, CGA stopped the process that turns on inflammation-related genes. Our findings match a recent study that showed CGA's positive effect in rats with spinal injuries (Kitazume-Taneike *et al*., 2019). Toll-like receptors (TLRs) represent a distinct subset of transmembrane proteins that play a pivotal role in modulating the mechanisms associated with inflammation and the innate immune response (Kumar, 2020). These receptors serve as primary sensors in detecting and responding to various external and internal stimuli that can pose threats to the host organism. TLR4, one of the most studied members of this family, is adept at recognizing both endogenous and exogenous molecular signatures. Specifically, it can detect danger-associated molecular patterns (DAMPs) which arise from cellular stress, damage, or death, and pathogen-associated molecular patterns (PAMPs) that are typical structures found in pathogens such as bacteria and viruses (Ni *et al*., 2020).

Upon identification of these molecular patterns, TLR4 undergoes a cascade of intracellular signalling. An integral component of this signalling process is its interaction with the MYD88 protein. Once TLR4 and MYD88 associate, they act as a catalyst to activate the NF-kB pathway, which in turn initiates a series of events that propagate a pro-inflammatory response within the host (Naibey *et al*., 2021). This response is essential for tackling infections and tissue damage but can also contribute to pathological conditions if left unchecked. In the context of our research, we scrutinized the expression patterns of both TLR4 and MYD88 within the hippocampal region of rats that had been induced with sepsis. We discerned those septic conditions instigated a marked upregulation of both these proteins, suggesting an amplified inflammatory response in the affected region. However, when the septic rats were administered CGA, there was a notable reduction in the expression of TLR4 and MYD88. This implies that CGA possesses the capability to modulate and potentially mitigate the heightened inflammatory signalling observed in sepsis.

Our findings align with Gu *et al*. (2021), who discovered that CGA can reduce liver inflammation by affecting the TLR4/MyD88/NF-κB pathway. In summary, our study shows that CGA can significantly decrease the release of TNF-α and IL-1β in the brains of septic rats. Additionally, CGA lowers the levels of TLR4 and MyD88 and stops NF-κB activity in these rats. This means that CGA's ability to reduce inflammation in septic conditions might be due to its impact on the TLR4/MyD88/NF-κB signalling pathway.

Oxidative stress, characterized by the overproduction of reactive oxygen species (ROS), is central to brain damage in sepsis. This stress damages lipids and proteins, resulting in neuron death and cellular harm, especially within structures like mitochondria and cell membranes (Della Giustina *et al*., 2020). Indicators of this oxidative stress, such as increased levels of malondialdehyde (MDA) and protein carbonyl groups, alongside decreased antioxidant enzyme activities like superoxide dismutase (SOD) and catalase (CAT), have been observed in septic rat brains (Metwally *et al*., 2020). Our research revealed that administering CGA substantially diminished oxidative products and boosted antioxidant enzyme activities in the hippocampus, the brain region crucial for memory and learning. This aligns with previous studies showcasing CGA's role in combating oxidative stress (Hassan *et al*., 2020).

To understand memory impairment, we utilized the open field habituation and the novel object recognition test. Our results highlighted inflammation and oxidative harm in the hippocampus post-CLP, with evidence suggesting that defending the hippocampus from oxidative damage can improve learning and memory post-CLP (Petronilho *et al*., 2020; Shi *et al*., 2023). Notably, CGA administration to rats effectively countered the cognitive dysfunction from CLP. The mechanism likely lies in CGA's capacity to curtail both inflammation and oxidative damage in the hippocampus.

In sum, our investigation underscores CGA's potential in reducing oxidative and inflammatory damage in the hippocampus of CLP-induced rats, leading to improved cognitive function. This advocates for deeper exploration into CGA's role in treating sepsis-related brain injuries, paving the way for its potential therapeutic application in clinical scenarios.

Conflict of Interest: There was no conflict of interest.

Authors contribution: QL planned and executed the study. QYZ, XC performed data analysis and wrote the manuscript. QL modified and revised the study before submission. All authors approved final proof.

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