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β -Casomorphin-7 Ameliorates Sepsis-Induced Acute Kidney Injury by Targeting NF- κ B Pathway

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The aim of this study was to investigate the protective effect of β -casomorphin-7 (β -CM-7) and its possible mechanisms on acute kidney injury (AKI).

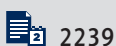
Material/Methods: Rats were randomly divided into a sham group, a cecal ligation and puncture (CLP) group, and a CLP+ β -CM-7 group. Kidney index, kidney function, and histopathology changes were assessed. The expression of neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (Kim-1), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha ($\text{I}\kappa\text{B}\alpha$), and p- $\text{I}\kappa\text{B}\alpha$ in kidney tissues were detected by Western blotting. Inflammatory and oxidative stress factors were detected by ELISA kits.

Results: The results showed that treatment with β -CM-7 reduced the levels of creatinine (Cre), blood urea nitrogen (BUN), NGAL, and Kim-1 induced by CLP, weakening the pathological damage. In the CLP + β -CM-7 group, the tumor necrosis factor- α (TNF- α) level and the DNA-binding activity of NF- κ B p65 were significantly reduced and the interleukin-10 (IL-10) level was significantly increased compared with the CLP group. β -CM-7 decreased the expression of p- $\text{I}\kappa\text{B}\alpha$ / $\text{I}\kappa\text{B}\alpha$. In addition, β -CM-7 increased the activity of superoxide dismutase (SOD) and decreased the level of malondialdehyde (MDA) in kidney tissue.

Conclusions: β -CM-7 attenuated sepsis-induced AKI through reducing inflammation and oxidative stress and by inhibition of nuclear factor (NF)- κ B activities. This study provides a new therapeutic agent for attenuating sepsis-induced kidney injury.

MeSH Keywords: **Acute Kidney Injury • Inflammation • Oxidative Stress**

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Background

Acute kidney injury (AKI) is a syndrome characterized by a sudden loss of kidney function and is associated with high mortality and morbidity rates [1,2]. Although the search for specific therapies to attenuate AKI or expedite recovery has attracted much attention, the prognosis of AKI is still poor [3]. The causes of AKI are various and complicated. Based on data from clinical studies, sepsis is the main cause of AKI, and about 50% of AKI cases are due to sepsis [4]. Sepsis-induced AKI results in high mortality, prolonged hospital stay, and increased cost of care. Thus, new therapeutic and preventive approaches for sepsis-induced AKI are crucial.

Inflammation is reported to be central common events in the pathogenesis of AKI [5, 6]. During sepsis, repeated stimulation of the kidney results in production of many pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α), along with the rapid release of large amounts of anti-inflammatory cytokines such as interleukin-10 (IL-10) [7]. TNF- α and IL-10 are associated with infection-induced immune responses. Previous studies have shown that the nuclear factor (NF)- κ B signaling pathway plays a crucial role in inflammation and can contribute to organ damage and mortality in patients with sepsis [8,9]. Moreover, sepsis-induced local or distant organ injuries are mediated by production of free radicals [10]. Therefore, early and timely interventions for inflammation and oxidative stress in kidney tissue may offer effective protection against sepsis-induced AKI.

β-casomorphin-7 (β-CM-7), an opioid peptide derived from food proteins, was first isolated from enzymatic digestion of bovine β-casein [11]. It is composed of 7 amino acids and has numerous biological activities, including reducing fasting blood glucose, inhibiting oxidative stress, and increasing growth-related hormones [12,13]. Evidence has indicated that β-CM-7 can attenuate oxidative stress damage induced by hyperglycemic [14]. Therefore, in the present study, a septic rat model of AKI was established by cecal ligation and puncture (CLP) method and the protective effects of β-CM-7 were investigated. We observed that β-CM-7 inhibited AKI by anti-inflammation and anti-oxidative effects. These findings suggest that β-CM-7 has potential to become a target to attenuate AKI and accelerate kidney recovery.

Material and Methods

Animals

Male Sprague-Dawley rats with an initial body weight of 250±20 g (7–8 weeks old) were obtained from the Experimental Animal Center of Suzhou Aiermaite Technology Co. (Suzhou, Jiangsu, China). Rats had free access to food and water and

were housed in an environment with temperature of 22±1°C and 50±10% humidity with a 12-h light/dark cycle. All experimental protocols were approved the Animal Ethics Committee of Nanjing Medical University.

Animal model establishment and treatments

The septic rat model with AKI was induced according to previously published protocols [15]. Rats were anesthetized with chloral hydrate (400 mg/kg) and the cecum was exposed through a ventral midline incision 1–1.5 cm long. Then, the cecum was ligated with a 4-0 silk at 1 cm to the distal end, and double punctures were made to extrude feces into the abdominal cavity. Finally, the cecum was placed back into the abdominal cavity and the abdomen was closed. Sham-operated rats underwent opening of the cecum without ligation puncture. The rats were resuscitated by intraperitoneal injection of 0.9% saline (24 mL/kg body weight) and returned to a warm cage.

Forty-eight rats were randomly divided into 3 groups: a sham group, a CLP group, and a CLP + β-CM-7 group (16 rats in each group). At 2 h after the surgery, rats in the CLP + β-CM-7 group received intraperitoneal injection of β-CM-7 (7.5×10^{-8} mol/d, Sigma-Aldrich, USA) [16]. Rats in the sham and CLP groups received equal volumes of physiological saline. To obtain the urine samples at 6 h and 24 h, 6 rats in each group were placed individually in metabolic cages to collect urine within 6 h of the preset time point. At 6 h and 24 h after treatment, other rats in each group were sacrificed and blood and kidney tissue samples were collected for biochemical quantification.

Determination of serum and urine biochemical indicators

The levels of creatinine (Cre) and blood urea nitrogen (BUN) in serum were measured using an AU 5800 automatic biochemistry analyzer (Beckman Coulter, Inc., USA) according to the manufacturer's instructions. The concentrations of TNF- α and IL-10 in serum and neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (Kim-1) in urine were measured by ELISA kits (Wuhan Boshi Biotechnology Co., Wuhan, Hubei Province, China) according to the manufacturer's instructions.

Kidney index

Kidney tissues were harvested, cleaned, washed in ice-cold normal saline, and weighed. The kidney index was calculated as: kidney weight (mg)/the body weight (g)×100%. Normally, the ratio of kidney to body weight is relatively constant. The weight of the damaged kidney can change and the kidney coefficient changes accordingly. An increased kidney coefficient shows organ congestion, edema, or hypertrophy; and a decreased kidney coefficient shows kidney atrophy and other degenerative changes.

Histological evaluation

Kidney segments were embedded in paraffin, then cut into 4-μm – thick sections, fixed in 10% (v/v) phosphate-buffered formalin for 24 h, and stained with hematoxylin and eosin (HE) solution. A light microscope (Leica DM LB2; Leica, Wetzlar, Germany) was used to observe the pathological changes.

The DNA-binding activity of NF-κB p65 detection

Nuclear extracts of kidney homogenates were prepared with a nuclear extract kit (Abcam, Inc., Cambridge, UK). The DNA-binding activity of NF-κB p65 was measured utilizing the ELISA-based NF-κB p65 transcription factor assay kit (Abcam, Inc., Cambridge, UK) according to the manufacturer's instructions.

Western blotting analysis

Kidney tissues from different groups were homogenized in RIPA lysis buffer containing EDTA-free protease inhibitor cocktail, and then were centrifuged at 15 000 rpm for 15 min. The supernatants were collected and total protein concentration was determined using a bicinchoninic acid (BCA) kit (Chengdu Must Biotechnology Co., Chengdu, China). Protein samples (20 μg) were subsequently subjected to 12% SDS-PAGE gels and transferred to a nitrocellulose membrane. After blocking with 5% non-fat dry milk at room temperature for 2 h, the primary antibodies of NGAL (1: 2000, Cell Signaling Technology, MA, USA), Kim-1 (Cell Signaling Technology, USA), caspase-3 (1: 1000, Cell Signaling Technology, MA, USA), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) (1: 1000, Cell Signaling Technology, MA, USA), p-IκBα (1: 1000, Cell Signaling Technology, MA, USA), and β-actin (1: 1000, Cell Signaling Technology, MA, USA) were added and incubated overnight. Subsequently, membranes were washed in TBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies. Protein bands were detected by enhanced chemiluminescent reagents (Amersham, Sydney, Australia).

Measurements of kidney oxidative stress

Kidney homogenates were prepared. The malondialdehyde (MDA) content and superoxide dismutase (SOD) level in kidney tissues were measured by ELISA kits (Nanjing Jiancheng Co., Nanjing, China) according to the manufacturer's instructions.

Statistical analysis

SPSS18.0 was used for statistical analysis and the data are reported as the mean ±SD. The differences between groups were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple range test. The level of statistical significance was set at p<0.05.

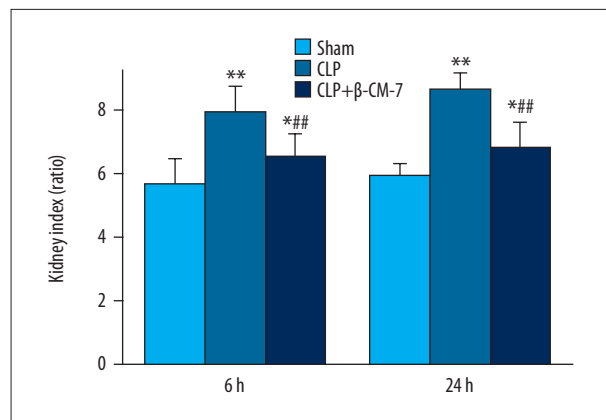


Figure 1. Effects of β-CM-7 on kidney coefficient (8 rats in each group). Data are presented as mean ±SD. * p<0.05, ** p<0.01 vs. sham group, ## p<0.01 vs. CLP group.

Results

Effects of β-CM-7 on kidney index

As shown in Figure 1, the kidney index in the CLP group significantly increased compared with the sham group at 6 h (from 5.81±0.81 to 7.80±0.70, p<0.01) and 24 h (from 5.91±0.71 to 8.35±0.42, p<0.01). However, administration with β-CM-7 significantly reduced the kidney index (from 7.80±0.70 to 6.38±0.55 at 6 h and from 8.35±0.42 to 6.89±0.57 at 24 h, p<0.01).

β-CM-7 ameliorates kidney damage

SCr and BUN levels in serum and NGAL and Kim-1 levels in urine and kidney tissues were used to evaluate kidney function (Figures 2A–2D, 3A–3D). The increased levels of Cre, BUN, NGAL, and Kim-1 showed in rats suffered from CLP in comparison with the sham group at 6 and 24 h, while β-CM-7 treatment reduced the levels of Cre, BUN, NGAL, and Kim-1 induced by CLP. These results show that β-CM-7 ameliorates kidney damage induced by CLP.

Effects of β-CM-7 on kidney histopathologic changes

As shown in Figure 4A, there were no histopathological abnormalities. In the CLP group kidney tissues were obviously damaged, and various histopathological alterations, including edema, inflammatory cell infiltration, glomerular enlarging, and narrow renal tubules, as well as swelling, degenerating, and falling of epithelial cells, were observed (Figure 4B). However, β-CM-7 treatment markedly attenuated these changes and preserved the renal architecture (Figure 4C).

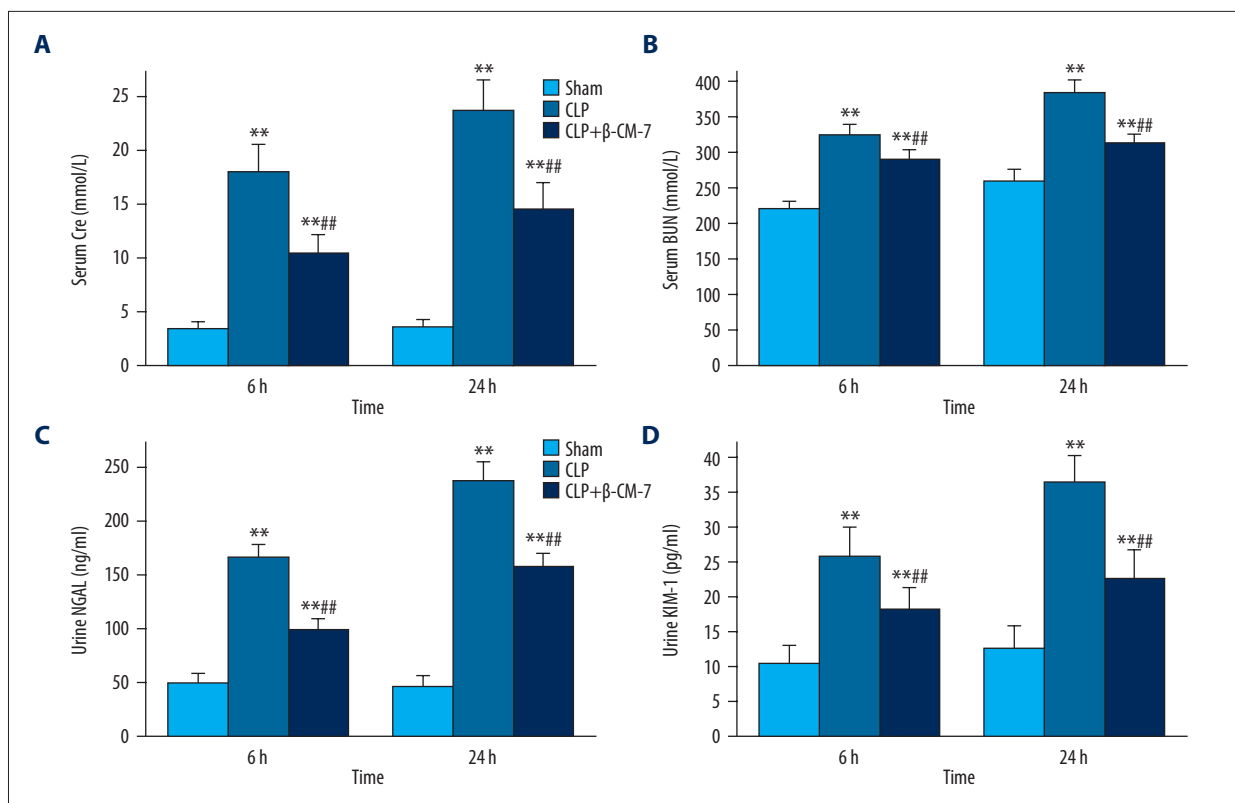


Figure 2. Effects of β -CM-7 on kidney injury related indicators (8 rats in each group). (A) Cre; (B) Serum BUN; (C) Urine NGAL; (D) Urine KIM-1. Data are presented as mean \pm SD. ** $p < 0.01$ vs. sham group, ## $p < 0.01$ vs. CLP group.

β -CM-7 inhibits inflammatory factors

As shown in Figure 5, CLP caused a significant increase in TNF- α level, while it caused a significant decline in IL-10 level at 6 and 24 h. β -CM-7 treatment significantly decreased the TNF- α level and increased the IL-10 level. The results show that β -CM-7 can attenuate the severity of inflammatory responses induced by CLP.

β -CM-7 prevents the DNA-binding activity of NF- κ B p65

As shown in Figure 6, the DNA-binding activity of NF- κ B p65 was strongly promoted in the CLP group in comparison to the sham group at 6 and 24 h. However, the elevation of NF- κ B p65 DNA-binding activity was greatly inhibited by β -CM-7 treatment. Activation of the NF- κ B signaling pathway was mitigated by β -CM-7 by suppressing the nuclear translocation.

β -CM-7 decreased the expression of p-I κ B α /I κ B α

As shown in Figure 3B, the expression of p-I κ B α /I κ B α was obviously increased in the CLP group in comparison to the sham group. However, the expression of p-I κ B α /I κ B α was greatly decreased by β -CM-7 treatment and the expression of NGAL and Kim-1 were decreased by β -CM-7 treatment. Those results

show that β -CM-7 ameliorates kidney damage by regulating the NF- κ B signal pathway.

β -CM-7 inhibits oxidative stress

As shown in Figure 7, in comparison with the sham group, the MDA content in the CLP group was significantly higher and the SOD levels were significantly lower at 6 and 24 h. After treatment with β -CM-7, the SOD level showed a marked increase compared with the CLP group, while the content of MDA had a significant decrease. The results show that β -CM-7 decreases the oxidative stress damage induced by CLP.

Discussion

The prognosis of AKI remains unsatisfactory because of the protean nature of septic AKI [17]. The CLP rat model is similar to a polymicrobial infection of intestinal origin in humans, so it was established for investigating the effect of β -CM-7 on sepsis-induced AKI [18]. We revealed the protective effect of β -CM-7 against sepsis-induced AKI in rats. These beneficial actions were mediated via reducing oxidative stress, attenuating inflammation, and inhibiting NF- κ B.

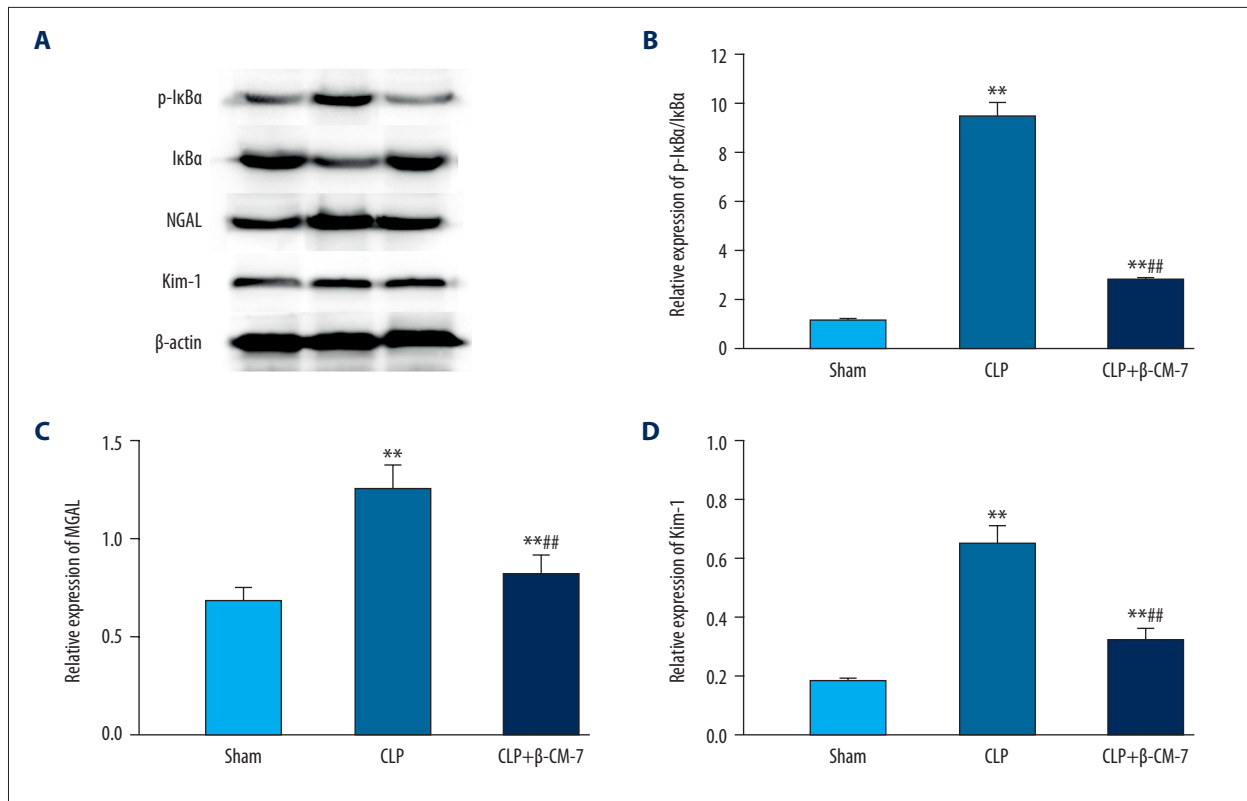


Figure 3. (A–D) Effects of β-CM-7 on NGAL and Kim-1 levels and the expression of p-IκBα/IκBα in kidney tissues (4 rats in each group). Data are presented as mean ±SD. ** p<0.01 vs. sham group, ## p<0.01 vs. CLP group.

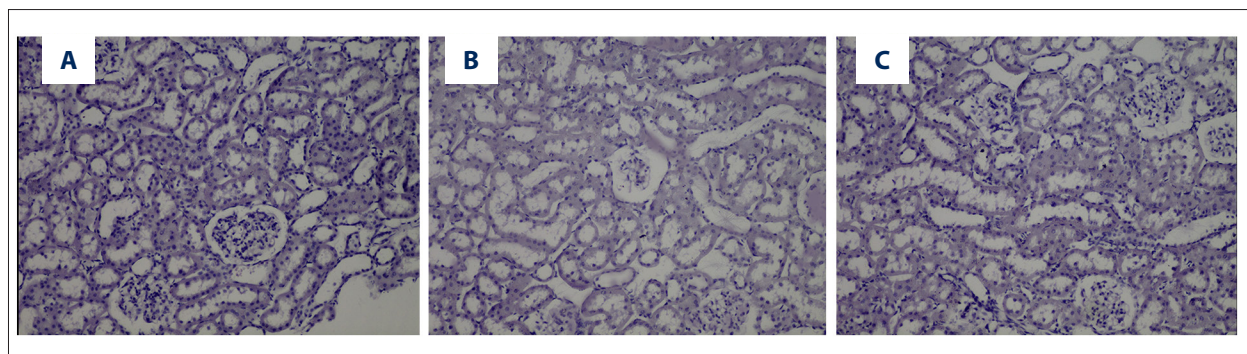


Figure 4. Effects of β-CM-7 on kidney histopathologic changes (4 rats in each group). (A) Sham group, (B) CLP group, (C) CLP + β-CM-7 group.

Cre and BUN in serum and KIM-1 and NGAL in urine are the classical index to evaluate kidney function. We showed that rats in the CLP group had substantial kidney injury with distinct changes in Cre and BUN in serum and KIM-1 and NGAL in urine, as well as increased kidney index. Our study shows that β-CM-7 attenuated the increased Cre and BUN in serum, as well as KIM-1 and NGAL in urine, which indicates that β-CM-7 improves renal function in septic AKI rats. Histological evaluation of kidney tissue further supports those results. Histopathological changes, including edema, inflammatory cell infiltration, glomerular enlarging, and narrow renal tubules, as

well as the swelling, degeneration, and detachment of epithelial cells were observed in the CLP group, and β-CM-7 attenuated the histological changes. These results demonstrate that β-CM-7 protects against sepsis-induced AKI.

The systemic immune responses mediated by pro- and anti-inflammatory mediators are associated with the pathophysiology of AKI [19,20]. The essence of sepsis is systemic inflammatory reactions, and many inflammatory mediators and enzymes take part in the process of injury. TNF-α is an important pro-inflammatory cytokine that is a marker

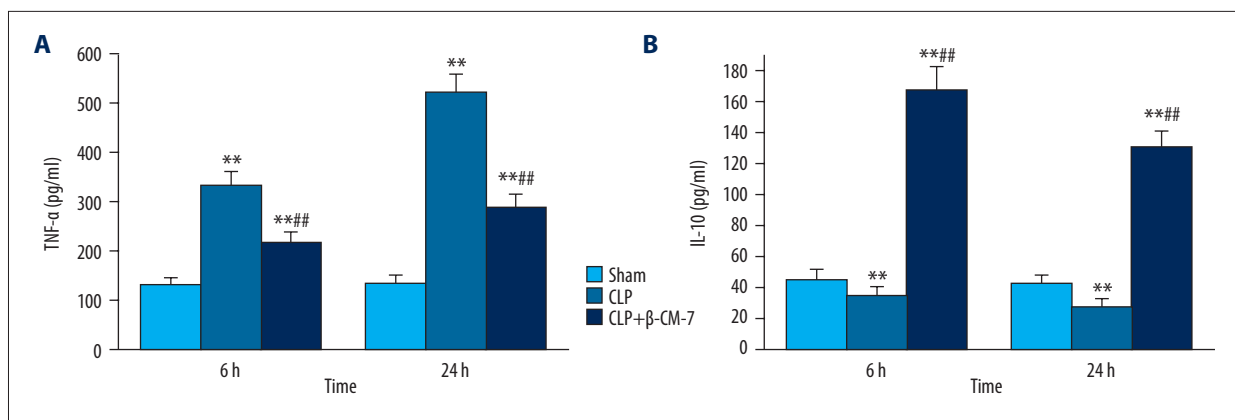


Figure 5. Effects of β-CM-7 on the level of TNF-α and IL-10 in serum (8 rats in each group). (A) TNF-α, (B) IL-10. Data are presented as mean ±SD. ** p<0.01 vs. sham group, *** p<0.01 vs. CLP group.

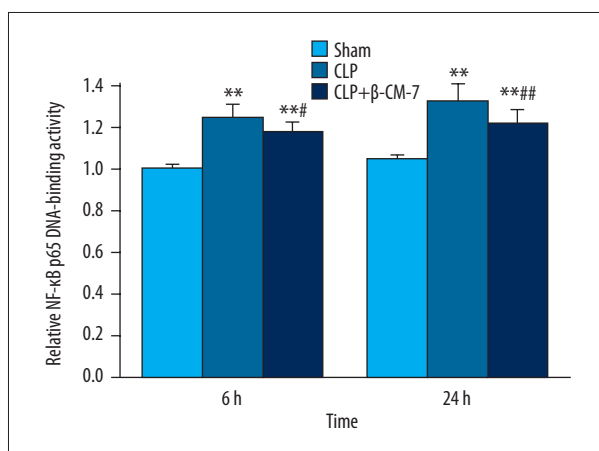


Figure 6. Effects of β-CM-7 on DNA-binding activity of NF-κB p65 (4 rats in each group). Data are presented as mean ±SD. ** p<0.01 vs. sham group, *** p<0.01 vs. CLP group.

of AKI severity [21]. IL-10, one of the primary anti-inflammatory cytokines, appears to limit and control inflammatory responses. In this study, in the CLP group, we found that the level of pro-inflammatory cytokines was increased and anti-inflammatory cytokines was decreased. However, after β-CM-7 intervention, the rise of TNF-α was inhibited, along with the increase of IL-10. These results indicate that β-CM-7 reduces sepsis-induced kidney injury through decreasing TNF-α level and increasing IL-10 level.

The NF-κB signaling pathway plays a key role in inflammatory reactions and immune responses [22]. Numerous animal experiments have confirmed that NF-κB is pivotal in the pathophysiology of sepsis and sepsis shock [23,24]. Under normal physiological conditions, NF-κB is sequestered in the cytoplasm by its inhibitor IκB [25]. When stimulated, it is activated by phosphorylation of IκB via translocation of NF-κB subunits to the nucleus [26]. The present study demonstrates that CLP stimulation dramatically increases the DNA-binding activity of NF-κB p65 and the expression of p-IκBα/IκBα. However, β-CM-7 treatment considerably decreased the DNA-binding activity of

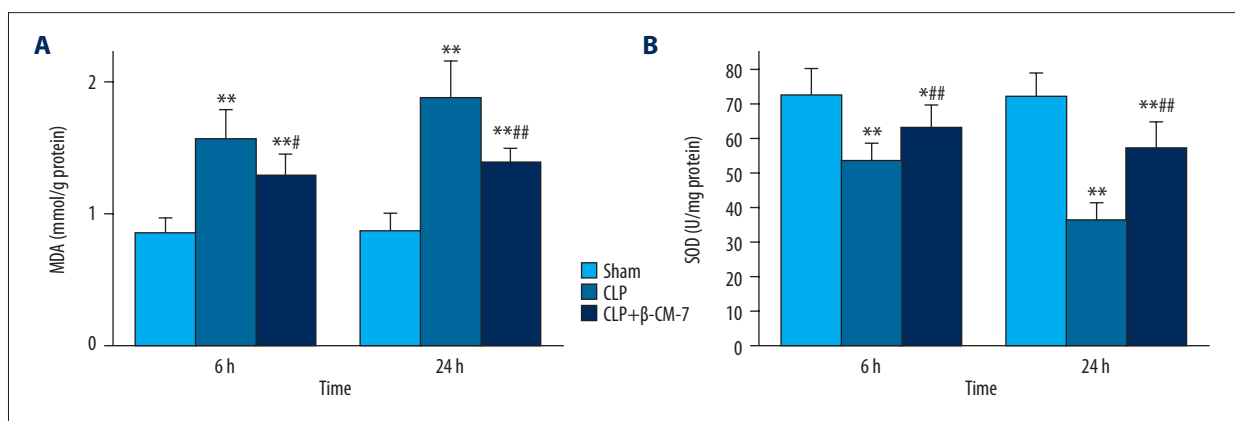


Figure 7. Effects of β-CM-7 on MDA content and SOD level in kidney tissues (4 rats in each group). (A) MDA, (B) SOD. Data are presented as mean ±SD. ** p<0.01 vs. sham group, *** p<0.01 vs. CLP group.

NF-κB p65. These findings demonstrate that blocking NF-κB signaling pathways might underlie β-CM-7 abatement of kidney inflammation.

Furthermore, we confirmed the suppressive effect of β-CM-7 on oxidative stress induced by CLP surgery. The involvement of oxidative stress in the pathogenesis of sepsis-induced AKI is well established in the literature [27]. Numerous studies have reported that the free radical reactions were pathologically exacerbated in septic patients, and the balance between oxidation and antioxidation was obviously disturbed [28,29]. MDA, the foremost representative lipid peroxidation, is a hallmark of oxidative stress. SOD is the primary line of defense against tissue damage [30]. We found that β-CM-7 promoted the activity of SOD and remarkably inhibited the reduced level of

MDA. These results show that β-CM-7 reduces sepsis-induced kidney damage by inhibiting oxidative stress.

Conclusions

We demonstrated that β-CM-7 can ameliorate sepsis-induced kidney injury. These beneficial actions were mediated via suppression of kidney inflammation and oxidative stress, inhibiting activation of the NF-κB signaling pathway. Our results suggest that β-CM-7 could be a new therapeutic agent for attenuating sepsis-induced kidney injury.

Source of support

None.

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