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Original Article

Assessment of inflammatory markers and mitochondrial factors in a rat model of sepsis-induced myocardial dysfunction

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Abstract: The present study aimed to investigate the expression of inflammatory markers and mitochondrial function-related genes, as well as their temporal relationship with cardiac myocyte injury in a rat model of sepsis. The sepsis model was constructed using cecal ligation and puncture (CLP). Two hours after CLP, the levels of inflammatory cytokines (interleukin [IL]-1β, IL-6, and TNFα) and myocardial function markers (serum brain natriuretic peptide [BNP], cardiac troponin-I [cTNI], and procalcitonin [PCT]) were increased significantly, falling from around 9 hours postoperatively. The concentration of nitric oxide (NO) in the heart tissue was increased 6 hours after CLP. The heart rate (HR) of rats that underwent CLP decreased 2 hours after surgery and then increased to above-normal values. The left ventricular short axis shortening (FS) and left ventricular ejection fraction (LVEF) were decreased at 2 hours postoperatively and reached a minima at 6 hours. Stroke volume (SV), cardiac output (CO), and changes and heart index (CI) results indicated myocardial dysfunction. Western blot analysis demonstrated the increased expression of mitochondrial function-related proteins and activation of mitochondrial apoptotic pathways. Hematoxylin and eosin staining and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays revealed that the proportion of proapoptotic cells was significantly higher in rats that underwent CLP than sham surgery at 2 to 24 hours postoperatively. Taken together, our results indicate that-in the rat model-CLP-induced sepsis leads to impaired cardiac function. Furthermore, induction of the expression of mitochondrial function-related genes indicated that myocardial cell mitochondrial function was disrupted, further aggravating cardiomyocyte apoptosis. These results provide a theoretical basis for the treatment of sepsis-induced myocardial dysfunction.

Keywords: Inflammation, mitochondria, myocardial dysfunction, sepsis

Introduction

Myocardial dysfunction is a common complication of sepsis, for which the mortality rate remains as high as 30.8% despite the production of the sepsis management guidelines by the Surviving Sepsis Campaign [1]. It is estimated that over 50% of patients with sepsis experience symptoms of myocardial dysfunction [2], and sepsis is a major cause of death in critical care units. Nevertheless, the underlying mechanism of sepsis-induced myocardial dysfunction remains unclear.

There is evidence to suggest that cytokines are key factors in the pathology of sepsis-induced myocardial injury [3]. Studies have indicated the up-regulation of tumor necrosis factor (TNF) α and interleukin (IL)-1β to be associated with myocardial depression *in vitro* [4]. Anti-TNF therapy has been shown to improve the cardiac function of patients with sepsis [5], supporting the suggestion that cytokine activation is an important aspect of the etiology of sepsis-induced myocardial dysfunction.

Mitochondrial function is impaired during the progression of sepsis and septic shock, which may influence the severity and subsequent poor outcomes of sepsis [6, 7]. Serum markers of mitochondrial dysfunction have also been detected in human patients and rodents with sepsis-induced cardiomyopathy [8]. Reduced activity of enzyme complexes in the mitochon-

drial electron transport chain and production of reactive oxygen species have been observed in the hearts of animals with sepsis [9, 10]. Inhibiting the mitochondrial permeability transition has been shown to improve cardiac function in animal studies [11].

Peroxisome proliferator-activated receptor gamma (PPARγ) is a member of the PPAR family, which has been demonstrated to have anti-inflammatory effects. Its protective role in inflammation has been reported in the context of ischemia reperfusion injury and inflammatory response after tissue injury [12, 13]. Activation of PPARγ protects the brain against microvascular dysfunction during sepsis, reducing systemic inflammation [14]. However, the influence of changes in PPARγ expression on the induction of sepsis-induced myocardial dysfunction is not well studied.

Although several reports have suggested that the presence of inflammatory cytokines and mitochondrial dysfunction contribute to the injury of the myocardium, few studies have investigated the temporal relationship between expression of inflammatory cytokines and mitochondrial dysfunction. In the present work, we established a cecal ligation and puncture (CLP)-induced sepsis model in rats, by constructing a rat model, then measured the expression levels of molecules involved in the activation of inflammation and mitochondrial function, as well as the expression of proteins associated with myocardial injury. We investigated the temporal associations of these factors with the progression of myocardial injury.

Materials and methods

Rat model of cecal ligation and puncture-induced sepsis

Thirty adult male Sprague-Dawley rats (weight range, 250-300 g) were purchased from the Animal Experiment Center of Sun Yat-sen University (Guangzhou, China). Rats were housed with access to food and water *ad libitum*. Rats were maintained on a 12-hour dark/12-hour light cycle. For CLP-induced sepsis, rats were randomly assigned to one of five groups (n=6 per group). Rats assigned to the CLP groups (the CLP 2 hr, CLP 6 hr, CLP 9 hr, and CLP 24 hr group) were anesthetized by intraperitoneal injection of sodium pentobarbital (120 mg/kg) as previously described [15]. An abdominal wall incision was made under aseptic conditions to expose the cecum, and the cecum was ligated below the ileocecal valve. A 21-gauge needle was used to perforate the cecum 10 times, and some of the feces were allowed to enter the abdominal cavity by gently pressing on the cecum. The cecum was reattached and the abdominal skin sutured. As a sham group, the same abdominal incision and cecal operation were performed but without cecal ligation or puncture. Rats were anesthetized with sodium pentobarbital and then euthanized to obtain heart tissues at 2, 6, 9, and 24 hours after CLP. The study and all procedures were approved by the Animal Care and Use Committee of the Shenzhen University (approval number 2017-0524004).

Isolation of RNA and quantitative reverse transcriptase polymerase chain reaction

The hearts of CLP and sham rats were excised at the time points indicated and frozen with liquid nitrogen. Total RNA was isolated from heart tissues using TRIzol reagent (Invitrogen, CA, USA). Then, cDNA was synthesized using PrimeScript RT Master Mix (Takara, Dalian, China) with random primers and amplified using the SYBR real-time polymerase chain reaction (PCR) master mix (TOYOBO, Osaka, Japan) kit according to the manufacturer's instructions. Amplification was performed using an ABI 7000 Taqman system (Applied Biosystems, CA, USA). The relative expression of IL-1β, IL-6, and TNFα was quantified using the $2^{-\Delta\Delta Ct}$ method. Expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was analyzed as an internal control. The sequences of the primers used in this study were as follows: Rat-IL-6-F: 5'-TTGCCTTCTTGGGACTGATGT-3'; Rat-IL-6-R: 5'-ATATACTGGTCTGTTGTGGGTGGT-3'; RAT-TNFα-F: 5'-CGTCAGCCGATTTGCCATTT-3'; RAT-TNFα-R: 5'-TCCCTCAGGGGTGTCCTTAG-3'; Rat-PGC-1α-F: 5'-AGACCAGTGAACTACGGGA-3'; Rat-PGC-1α-r: 5'-AGAGCAAGAAGGCGACA-3'; Rat-IL-1β-F: 5'-CTGACAGACCCCAAAAG-3'; Rat-IL-1β-R: 5'-TCTCCACAGCCACAATG-3'.

Enzyme-linked immunosorbent assay and detection of nitric oxide

Peripheral blood was collected under anesthesia and then centrifuged at 3,000 × g for 30 minutes. The serum brain natriuretic peptide

(BNP) enzyme-linked immunosorbent assay (ELISA) kit (CSB-E07972r; Cusabio, Wuhan, China), cardiac troponin-I (cTNI) ELISA kit (LS-F23616-1; LifeSpan BioSciences, USA), procalcitonin (PCT) (CSB-E13419r; Cusabio), IL-1β (CSB-E08055r, Cusabio), IL-6 (CSB-E04640r, Cusabio), and TNFα (CSB-E11987r, Cusabio) were used to determine concentrations of the relevant proteins according to the manufacturer's instructions. Meanwhile, the nitric oxide (NO) content of cardiac muscle tissue was measured in strict accordance with the instructions of the NO assay kit (S0021, Beyotime Biotech, China).

Echocardiographic measurements

Rats were anesthetized at 2, 6, 9, and 24 hours after CLP surgery, and echocardiography measurements of the following parameters were carried out: left ventricular short axis shortening (FS), left ventricular ejection fraction (LVEF), stroke volume (SV), cardiac output (CO), and changes and heart index (CI). Briefly, a Vevo 2000 ultrasonic system (VisualSonics, Toronto, Canada) was used for two-dimensional (2D) echocardiographic imaging. A high-resolution probe was positioned in the peripheral papillary muscular short axis to obtain 2D images of the left ventricle, and monitoring was maintained for 10 heart cycles under ultrasonic guidance. We recorded data of heart rate (HR), left ventricular systolic and diastolic anterior wall thickness (Awsth and Awdth, respectively), left ventricular systolic and diastolic posterior wall thickness (Pwsth and Pwdth, respectively), left heart end-diastolic diameter (LVEDd), left ventricular end-systolic diameter (LVESd), left ventricular end-diastolic diameter (EXLVDd), and left ventricular short axis shortening rate (FS%). All measurements were performed by two lab technicians who were blinded to the experimental design.

Western blotting

Heart tissues resected from CLP or sham rats were frozen in liquid nitrogen, ground, and lysed in radioimmunoprecipitation assay (RIPA) lysis solution supplemented with 2 mM phenylmethylsulfonyl fluoride (PMSF). Protein concentrations were quantified by bicinchoninic acid assay (Thermo Fisher Scientific, MA, USA). A total of 30 μg protein was run on each lane of a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Gels were transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, CA, USA) after overnight incubation with primary antibodies specific for nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p-p65 (1:1,000, cat. #3033), NF-κB p65 (1:1000, #8242), PPARγ (1:500, SC7196), PPARγ coactivator (PGC)-1α (1:1,000, ST1202), mitochondrial transcription factor A (mtTFA; 1:1,000, ab138351), nuclear respiratory factor 1 (NRF-1; 1:10,000, ab1759-32), Casp-3 (1:1,000, #9662), and cleaved-Casp-3 (1:1,000, Asp175) at 4°C. Membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (cat. #115-035-044) for 1 hour at room temperature. Bands were visualized using Western blot enhanced chemiluminescence (ECL) substrate (Bio-Rad). Antibodies to GAPDH (1:8,000, cat. #9662) were applied to provide an internal reference.

Terminal deoxynucleotidyl transferase dUTP nick end labeling assay

Myocardial apoptosis was assessed using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Myocardial tissues were fixed with 4% paraformaldehyde then washed three times with phosphate-buffered saline (PBS), embedded in paraffin, sectioned, deparaffinized in xylene for 10 minutes, and deparaffinized again with fresh xylene for 10 minutes. Gradient ethanol dehydration was conducted by dipping sections in absolute ethanol for 5 minutes and then dipping in 90% ethanol for 2 minutes and then 70% ethanol for 2 minutes. After washing with distilled water for 2 minutes, 20 μg/ml of DNase-free proteinase K was added to each sample; then, samples were incubated at 37°C for 30 minutes. After three washes with PBS, 50 μl of the TUNEL reaction solution was added, and samples were incubated at 37°C for 60 minutes in the dark. Sections were then washed three times with PBS and subjected to Hoechst staining for 10 minutes, after which they were observed under a fluorescence inverted microscope (IX, Olympus, Japan). The total nuclei (stained blue) and TUNEL-positive nuclei (stained green) were counted in five randomly chosen fields. The rate of apoptosis was calculated from the number of the TUNEL-positive nuclei and exported to GraphPad for further analysis.

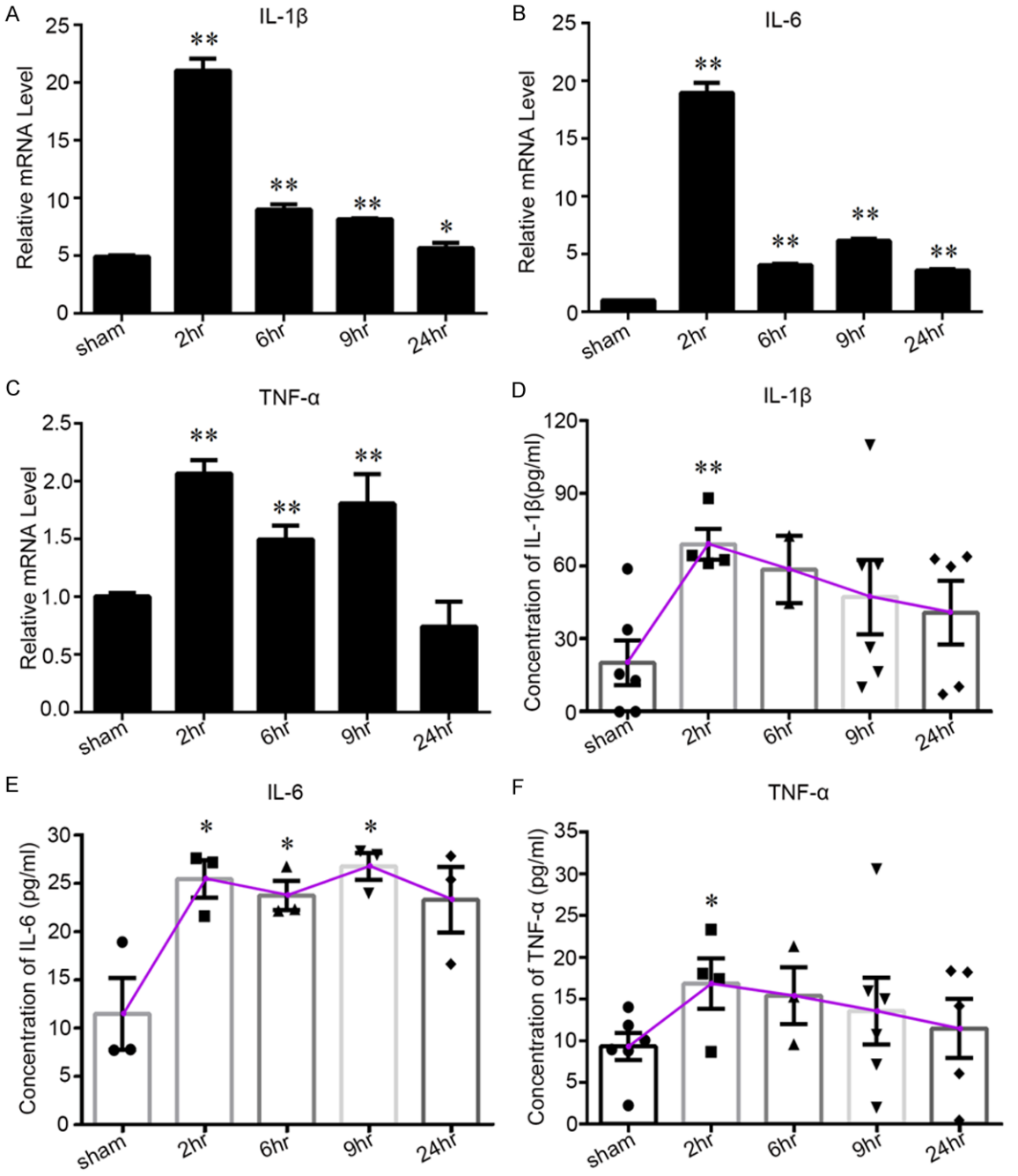

Figure 1. The expression levels of interleukins 1β and 6 and tumor necrosis factor α were determined at 2, 6, 9, and 24 hours after cecal ligation and puncture. A-C. Relative expression of IL-1β, IL-6, and tumor necrosis factor (TNF) α mRNA detected by quantitative reverse transcriptase polymerase chain reaction. D-F. Enzyme-linked immunosorbent assay revealed dynamic changes in the levels of IL-1β, IL-6, and TNFα in rats of the sham and cecal ligation and puncture groups after surgery. *P<0.05; **P<0.01 vs. sham.

Statistical analysis

All statistical analysis was performed using SPSS 19.0 software (IBM, USA). Data are presented as mean ± standard deviation (SD). The student's t-test was used to analyze differences between two groups, and one-way analysis of variance was used for comparisons between three or more groups. Differences were considered significant at P<0.05.

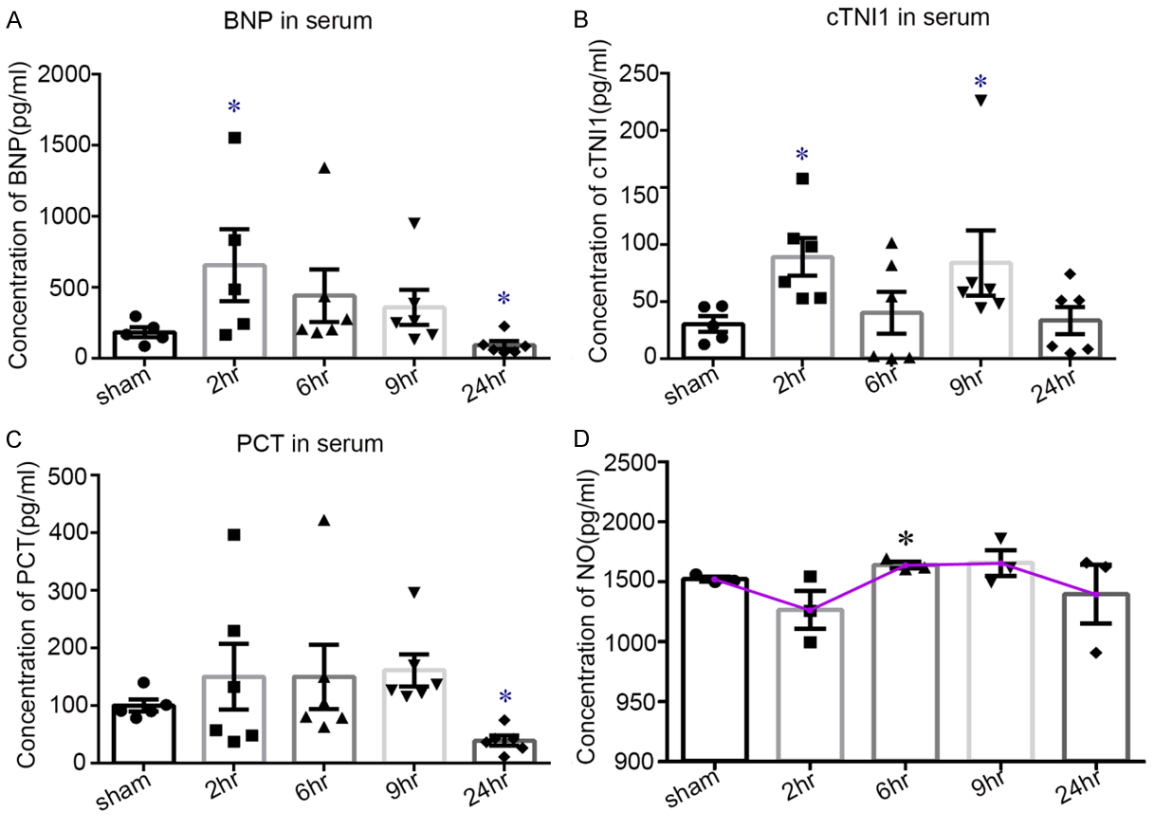

Figure 2. Serum concentrations of brain natriuretic peptide, cardiac troponin-I, and procalcitonin as well as expression of NO in myocardial tissues. Serum levels of (A) brain natriuretic peptide, (B) cardiac troponin-I, and (C) procalcitonin were measured in the sham and CLP groups by immunoluminometric and enzyme-linked immunosorbent assays at 2, 6, 9, and 24 hours postoperatively. (D) Concentration of NO was measured in myocardial tissues at 2, 6, 9, and 24 hours postoperatively. *P<0.05 vs. sham.

Results

Serum inflammation and cardiac function-related factors were induced 2 hours postoperatively

The level of IL-1β was significantly increased 2 hours after CLP induction, after which they decreased (**Figure 1A**), while mRNA levels of IL-6 and TNFα fluctuated (**Figure 1B**, **1C**). The ELISA results supported quantitative reverse transcriptase (qRT)-PCR results (**Figure 1D**, **1E**). The expression of TNFα (**Figure 1F**), BNP, cTNI1, and PCT increased 2 hours after CLP induction, after which they decreased. However, cTNI1 and PCT increased again 9 hours after cardiac injury (**Figure 2A-C**). The production of NO in cardiac muscle tissue decreased 2 hours after surgery and increased slightly after 6 hours (**Figure 2D**). These results confirm that severe myocardial injury occurred at 2 hours after surgery.

Cardiac function was suppressed 2 hours after surgery

Echocardiography revealed the HRs of CLP rats to be significantly higher than those of the sham group (**Figure 3A**). Clear images were obtained for all five experimental groups. The results of 2D ultrasonography showed that HR decreased in 2 hours after surgery, after which it increased significantly. The mean HRs of the CLP groups were significantly higher than the sham group (**Figure 3B** and **3C**). From 2 hours postoperatively, the SV decreased to reach a minima at 6 hours postoperatively, after which it gradually increased. However, the SV of experimental groups did not reach the same level as the sham group within 24 hours after surgery (**Figure 3D**). The LVEF values were consistent with the trend for FS (**Figure 3E**). The SV fluctuated over the 24 hours after surgery reaching a minima of lower than the sham group at 24 hours (**Figure 3F**). The results of CO

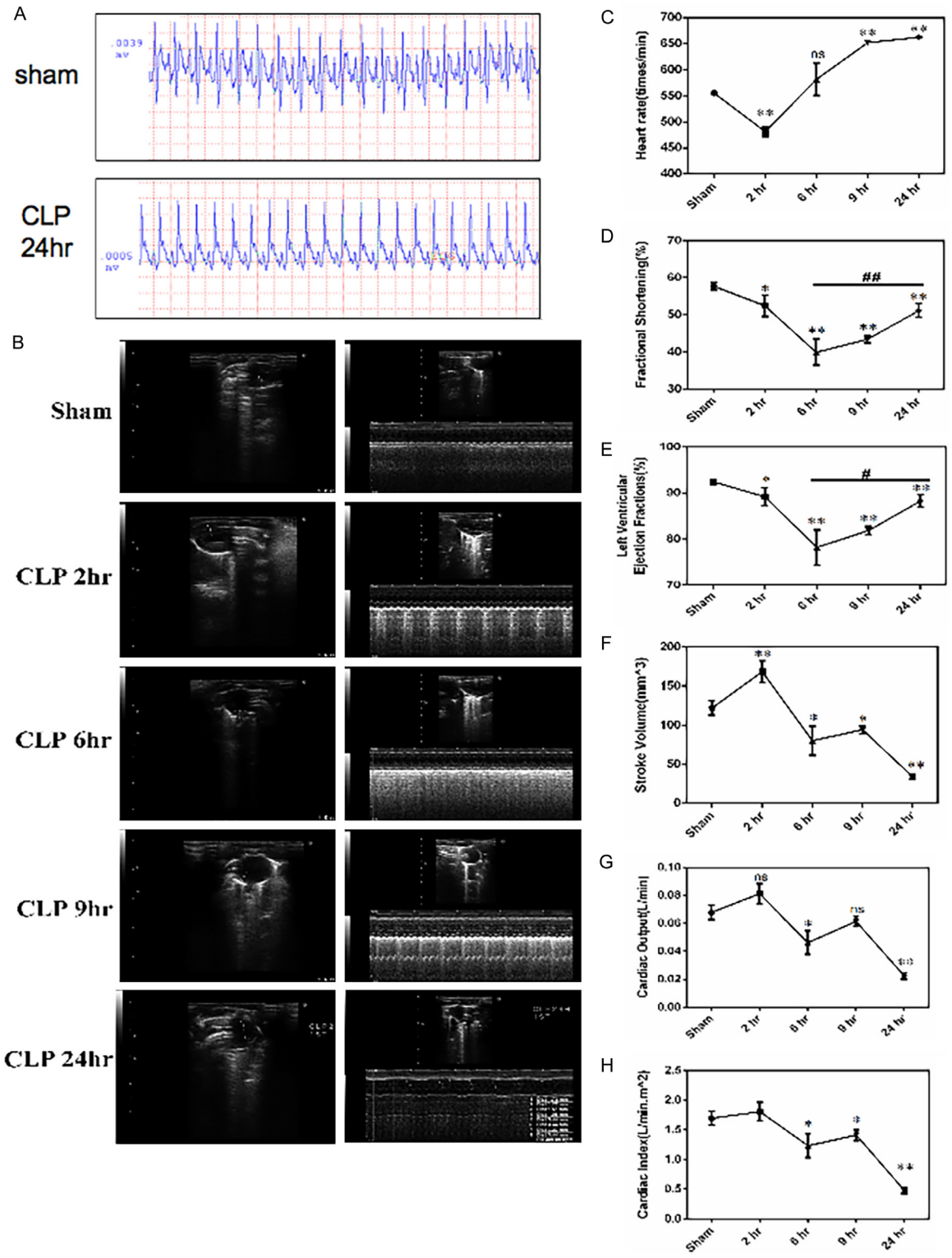

Figure 3. Evaluation of heart function evaluation in the sham and cecal ligation and puncture groups at 2, 6, 9, and 24 hours postoperatively. Representative images of (A) HR and (B) echocardiography results of rats in the sham and cecal ligation and puncture groups. Dynamic changes in (C) heart rate, (D) left ventricular short axis shortening, (E) left ventricular ejection fraction, (F) stroke volume, (G) cardiac output, and (H) CI of rats in the CLP group. Abbreviations: HR, heart rate; FS, left ventricular short axis shortening; LVEF, left ventricular ejection fraction; SV, stroke volume; CO, cardiac output; CI, changes and heart index. *P<0.05 and **P<0.01 vs. sham. Ns, no significance.

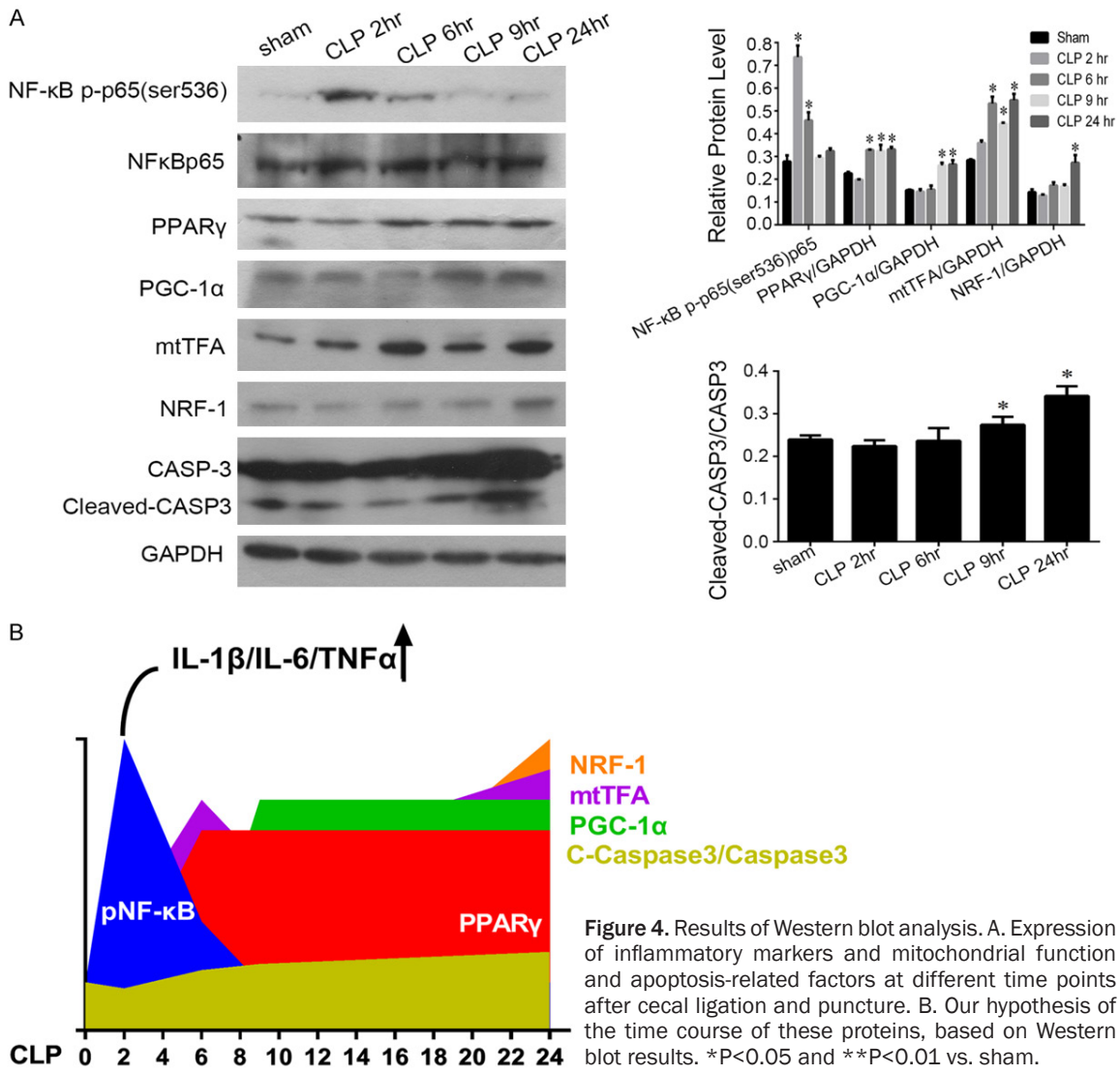

Figure 4. Results of Western blot analysis. A. Expression of inflammatory markers and mitochondrial function and apoptosis-related factors at different time points after cecal ligation and puncture. B. Our hypothesis of the time course of these proteins, based on Western blot results. *P<0.05 and **P<0.01 vs. sham.

showed that expression was down-regulated at 6 and 24 hours postoperatively but up-regulated at 2 and 9 hours without significant difference (**Figure 3G**). The CI exhibited a significant decrease at 6 hours postoperatively, decreasing to reach a minima at 24 hours (**Figure 3H**).

Mitochondrial function-related factors were activated 6 hours after cecal ligation and puncture on rats

Western blot analysis revealed that NF-κB p-p65 was activated 2 hours after CLP, while other proteins were up-regulated after 6 hours. Up-regulation of PPARγ was observed after 6 hours, which continued for 24 hours. The expression levels of PGC-1α and mtTFA were increased at 9 and 24 hours. The results of NRF-1 expression indicated significant up-regulation at 24 hours, although there was no significant difference on the sham group before this time point. Significant up-regulation of Casp-3, a key protein in mitochondrial apoptosis, was observed 9 hours postoperatively (**Figure 4A**). Western blot results were compared with the trends over time and were simulated using waveforms. The results reflected the expression of IL-1β, IL-6, and TNFα. The expression of NF-κB p-p65 peaked at 2 hours after activation and then decreased. After 2 hours, and most notably after 6 hours, the expression of PPARγ, PGC-1α, mtTFA, and NRF-1 was increased, whereas Casp-3 and cleaved-Casp-3 were only detected after 24 hours (**Figure 4B**).

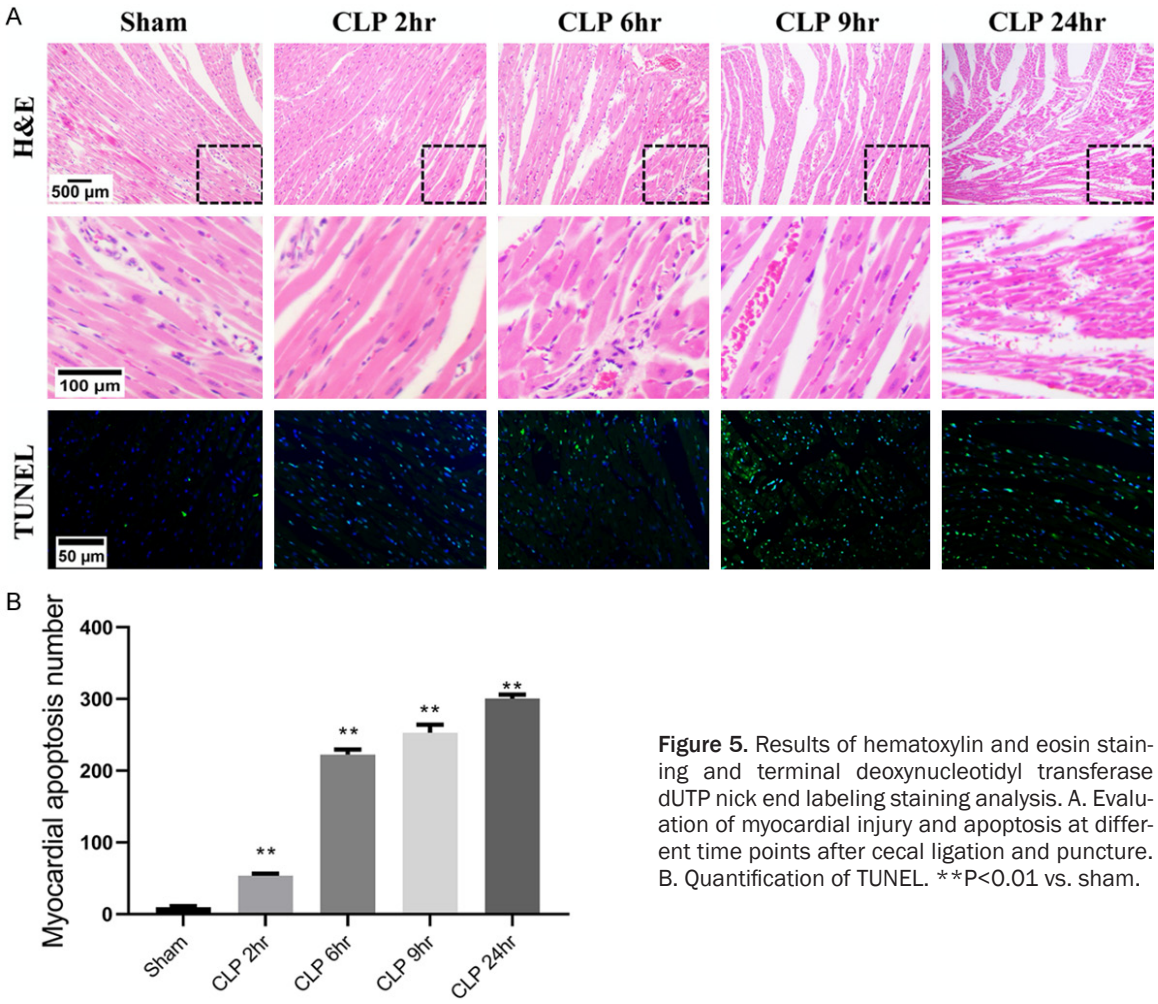

Figure 5. Results of hematoxylin and eosin staining and terminal deoxynucleotidyl transferase dUTP nick end labeling staining analysis. A. Evaluation of myocardial injury and apoptosis at different time points after cecal ligation and puncture. B. Quantification of TUNEL. **P<0.01 vs. sham.

Myocardial injury was observed 6 hours after cecal ligation and puncture

Analysis of hematoxylin and eosin staining revealed the arrangement of myocardial cells to be more disordered in cardiac muscle tissue from rats in the CLP groups compared with sham rats. After CLP surgery, the variability of cell morphology increased over time, and the arrangement and structure of cardiomyocytes changed significantly 6 hours postoperatively. The TUNEL assay did not detect a significant difference in the number of apoptotic cells at 2 hours after CLP, but this increased from 6 hours, becoming significant at 9 and 24 hours (**Figure 5A** and **5B**).

Discussion

This study investigated the changes in inflammatory factors (IL-1β, IL-6, and TNFα); myocardium function markers (TnI, BNP, and PCT); cardiac function; mitochondrial function; and expression of apoptosis-related proteins at 2, 6, 9, and 24 hours in a rat model of sepsis. Our findings provide evidence of the relationship between inflammation, inhibition of cardiac function, and changes in mitochondrial function over time. We found that inflammatory responses occurred after CLP; the expression of inflammatory cytokines increased 2 hours after injury, and the inflammatory transcription factor NF-κB p-p65 was activated. Myocardial injury markers were also up-regulated 2 hours after CLP. Electrocardiograms showed increased HR and suppressed cardiac function 6 hours after surgery. Western blots revealed the expression of PPARγ and PGC-1α to be up-regulated from 6 to 9 hours postoperatively, while the levels of NRF-1, Casp-3, and its activated form cleaved-Casp-3 were increased after 24 hours. The number of apoptotic cells was also increased.

At present, the pathogenesis of sepsis-induced cardiomyopathy remains unclear, although two possible mechanisms have been proposed. First, myocardial ischemia can lead to a reduction in coronary blood flow [16]. However, this hypothesis lacks evidence, and the more widely accepted mechanism is as follows: factors including endotoxins, cytokines, and NO are released into the blood. During sepsis, bacterial toxins can induce the release of inflammatory factors such as TNFα, IL-1, IL-6, and NO, directly or indirectly leading to the suppression of myocardial function [5, 17, 18]. However, this release of inflammatory factors produces a cascade-amplifying effect, stimulating the body to produce a large number of cytokines, with consequent induction of cardiac damage. Previous studies have shown an increase in cytokine levels within 2 hours of lung and kidney injury induced by acute sepsis [19]. The results of the present study support this, as we also observed activation of inflammatory cytokines at 2 hours post CLP.

The NF-κB family contains five transcription factors: Rel/p65, RelB, c-Rel, p50 (NF-κB1), and p52 (NF-κB32). The NF-κB protein binds to and phosphorylates the κB locus of a target gene, inducing the transcription of downstream genes [20]. As a nuclear transcription factor, NF-κB has been reported to regulate a variety of inflammation-related cytokines and to participate in the processes of immunity, inflammation, and apoptosis [21]. In this study, we found the activation of NF-κB p65 2 hours after CLP to reflect the expression of TNFα, IL-1, and IL-6. This activation of NF-κB p65 indicates that an inflammatory response has occurred. While PPARγ has been reported to play an important role in apoptosis of activated T cells during sepsis and function as an anti-inflammation and prognostic factor in sepsis [22], PGC-1α is essential for mitochondrial biogenesis. Once PGC-1α is activated, it activates NRF-1 and NRF-2 which subsequently induce the expression of mtTFA, a direct regulator of mitochondrial DNA replication and transcription [23-25]. Thus, the up-regulation of PPARγ, PGC-1α, NRF-1, and mtTFA that we observed 6 hours after CLP indicates that anti-inflammatory processes have been initiated and mitochondrial functions including mitochondrial biogenesis are altered at this time point.

Previous studies have suggested over-expression of inflammation factors such as signal transducer and activator of transcription 1 (STAT1), interferon regulatory factor 1 (IRF1), NF-κB, and TNFα could result in the activation of Casp-3 and contribute to cardiac dysfunction during sepsis, which may subsequently induce myocardial dysfunction [26, 27]. In the present study, we identified a fluctuating trend of first low then high expression of Casp-3 and cleaved-Casp-3 over the 24 hours following CLP, with the highest expression observed 24 hours postoperatively. We observed increased levels of cardiac damage and apoptosis in rats who underwent CLP, suggesting that the CLP-induced apoptosis and injury were enhanced from 6-24 hours postoperatively.

In summary, our results suggest that the activation of inflammatory pathways and induction of mitochondrial dysfunction and cardiac damage during the first 24 hours after CLP lead to the development of sepsis. Mitochondrial function of the cardiomyocytes is destroyed, mitochondrial oxygen conversion is reduced, and synthesis of adenosine triphosphate (ATP) is inhibited. These responses increase the burden on the heart, aggravate apoptosis, and cause irreversible damage to cardiomyocytes. We intend to continue research to confirm this hypothesis in the future.

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Disclosure of conflict of interest

None.

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