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Evaluation of cardiac troponin I in serum and myocardium of rabbits with experimentally induced polymicrobial sepsis

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Abstract: Sepsis is a potentially life-threatening condition, and it is frequently complicated by myocardial damage. Data on myocardial damage in rabbit caecal ligation and puncture (CLP) models are limited, although numerous animal models have been used to study sepsis-associated myocardial damage. This study aimed to investigate the effect of CLP on cardiac muscle by measuring serum cardiac troponin I (cTnI) concentrations and by detecting both histopathological changes and cTnI immunoreactivity in cardiomyocytes in rabbits. After CLP was performed in rabbits, blood samples were taken from the jugular vein at 0, 4, 8, and 12 h for haematological and biochemical analyses. At the end of the experiment, all of the rabbits were euthanised to examine the histopathological changes and the cTnI immunoreactivity in cardiac muscle tissue. No changes in serum cTnI concentration were observed in the experimental group (EG) or control group (CG) at 0 and 4 h. In EG, the mean serum cTnI concentrations were 0.230 ± 0.209 and 1.177 ± 0.971 ng/ml at 8 and 12 h, respectively. In CG, the mean serum cTnI concentrations were 0.032 ± 0.014 and 0.031 ± 0.021 ng/ml at 8 and 12 h, respectively. Moreover, cytoplasmic cTnI immunoreactivity decreased in EG compared with that in CG (P<0.01). The results demonstrated that CLP induced a systemic inflammatory response and caused myocardial damage in rabbits.
Key words: caecal ligation and puncture, cardiac troponin I, immunohistochemistry, rabbit, sepsis

Introduction

Sepsis is a clinical syndrome that is caused by a systemic inflammatory response to an infection, and myocardial damage is a common complication of sepsis [10, 36]. The mechanisms that underlie myocardial damage in sepsis include oxidative stress; activation of the synthesis of myocardial depressant factors, such as tumour necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), C5a; endotoxin; increased toll-like receptor (TLR) gene expression; elevated nitric oxide synthesis; impaired β-adrenergic signalling; changes in intracellular calcium trafficking; and mitochondrial dysfunction [3, 8, 17, 43].

Different experimental models have been developed to investigate the aetiology and pathogenesis of sepsis; these models include lipopolysaccharide (LPS) infusion, live pathogen inoculation, colon ascendens stent peritonitis, and caecal ligation and puncture (CLP) models [9, 33]. CLP is one of the most effective sepsis models, and it has been used to induce polymicrobial sepsis in rabbits [9, 13]. Use of the CLP model is advantageous, as it is an easy procedure, it identifies the presence of an infectious focus, it creates hyper and hypoinflammatory phases of sepsis, and it facilitates prolonged and reduced elevation of cytokine release [9].

Studies have investigated myocardial damage by determining changes in serum cardiac troponin I (cTnI) concentrations in both naturally occurring and induced sepsis. Elevated cTnI concentrations were observed in humans who were critically ill with sepsis [11]. In an-

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other study, elevated cTnI concentrations were found in calves after LPS infusion [29]. Elevated serum cTnI concentrations were also found in LPS-treated mice [44]. To the best of our knowledge, a limited number of studies have investigated the association between myocardial damage and cTnI concentrations in rabbit CLP models. Thus, this study aimed to investigate the effect of CLP on cardiac muscle by measuring serum cTnI concentrations and by detecting both histopathological changes and cTnI immunoreactivity in cardiomyocytes in rabbits.

Materials and Methods

Fourteen New Zealand rabbits purchased from the Firat University Experimental Research Center were used in this study. The rabbits were 3–6 months old and weighed 2.91 ± 0.26 kg. They were randomly divided into two groups: an experimental group (EG, n=8) and a control group (CG, n=6). All experiments were performed at the Department of Internal Medicine Laboratory at Firat University. The rabbits were reared at room temperature (20–22°C) and 60% humidity. This study was approved by the Ethics Committee for Experimental Animals of Firat University (13.01.2016, 2015/117).

CLP was performed on each of the 14 rabbits as previously described [13]. The rabbits were anaesthetised with an intramuscular injection of 5 mg/kg xylazine (Rompun®, Bayer, Leverkusen, Germany) and 25 mg/kg ketamine (Ketasol®, Richter Pharma AG, Vienna, Austria). Following administration of anaesthesia, a median laparotomy was performed. The caecum was removed from the abdominal cavity, and it was ligatured below the ileocaecal valve with 2/0 silk. Subsequently, an incision (approximately 1 cm) was made to the caecum below the ligation site, and the caecum contents were squeezed into the abdominal cavity. Finally, the caecum was returned into the abdominal cavity, and the abdominal wall was closed using sutures.

Blood samples were taken from the jugular vein of the rabbits in both groups for haematological and biochemical analyses at 0 (before experiment), 4, 8, and 12 h. After collection, the serum samples were centrifuged at 3,000 G for 10 min, and serum creatine kinase isoenzyme MB (CKMB), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and cTnI concentrations were measured on the same day. The serum samples were stored at −20°C for 2 months until determination of the serum C-reactive protein (CRP) concentration. The total WBC count and lymphocyte, heterophil, and monocyte ratios were detected manually. Serum CRP concentrations were measured using a rabbit-specific ELISA kit (FineTest, Wuhan Fine Biological Technology, Wuhan, Hubei, China) according to the manufacturer's instructions. Serum CKMB, AST, and LDH concentrations were measured using an Advia Centaur XP (Siemens Healthcare Diagnostics, Malvern, PA, USA) autoanalyzer. Serum cTnI concentrations were measured with an Advia Centaur TnI-Ultra assay (Siemens Healthcare Diagnostics). The Advia Centaur TnI-Ultra assay is a human-specific, three-site immunometric assay with chemiluminescence detection, and the capture antibody recognises the amino acids 27–40, whereas the detection antibody recognises the amino acids 41–49 and 87–91 [34]. The measurement range of this assay is 0.006–50 ng/ml according to the manufacturer.

At the end of the experiment, all of the rabbits were euthanised with an intravenous injection of 120 mg/kg ketamine (Ketasol®, Richter Pharma AG). Cardiac tissue samples were collected from the interventricular septum, right and left atrium, and ventricles. The samples were fixed in 10% formalin solution and then routinely processed and embedded in paraffin. Then, 3 μm-thick sections were stained with haematoxylin and eosin, and inflammatory changes were examined. Histopathological changes were scored semiquantitatively based on the severity of both degeneration/necrosis and cell infiltration by using a scale of 0 to 3 (0, no change; 1, mild; 2, moderate; 3, severe). The tissue samples were also examined for cTnI immunoreactivity by using a commercially available immunohistochemistry kit (ab64259, Abcam, Cambridge, UK). In brief, the process included the following steps: Three micrometre-thick serial sections were dewaxed in xylene and then hydrated using graded alcohols. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in methanol for 10 min. Subsequently, the sections were placed in citrate buffered saline in a microwave oven for 5 min. After washing with PBS for 5 min, the sections were incubated with monoclonal mouse anti-cTnI antibody (ab10231, Abcam) for 1 h at room temperature. After washing with PBS for 5 min, the sections were incubated with biotinylated goat anti-mouse immunoglobulin G (ab64259, Abcam) for 10 min at room temperature. After washing with PBS for 5 min, the sections were treated with streptavidin peroxidase complex (ab64259, Abcam). 3,3'-Diaminobenzidine was used as the chromogen, and Mayer's haematoxylin was used as the counterstain. Immunohistochemical scoring was performed semiquantitatively by determining the percentage of cTnI-positive cells in 10 different fields of each section.

Statistical analysis of all data was performed using SPSS 21 (IBM Corp., Armonk, NY, USA), and data are presented as the mean ± SD. Statistical differences be-

tween groups at different time points were evaluated with the Mann-Whitney U test. The two-tail Pearson test was used for correlation analyses. Significance was set at $P<0.05$.

Results

Figure 1 shows the changes in total WBC count; lymphocyte, heterophil, and monocyte ratios; and serum CRP concentrations after CLP. In EG, the lowest leukocyte count was $1.5 \pm 0.3 \times 10^3$ /μl at 4 h, and it remained low throughout the experiment. While the segmented

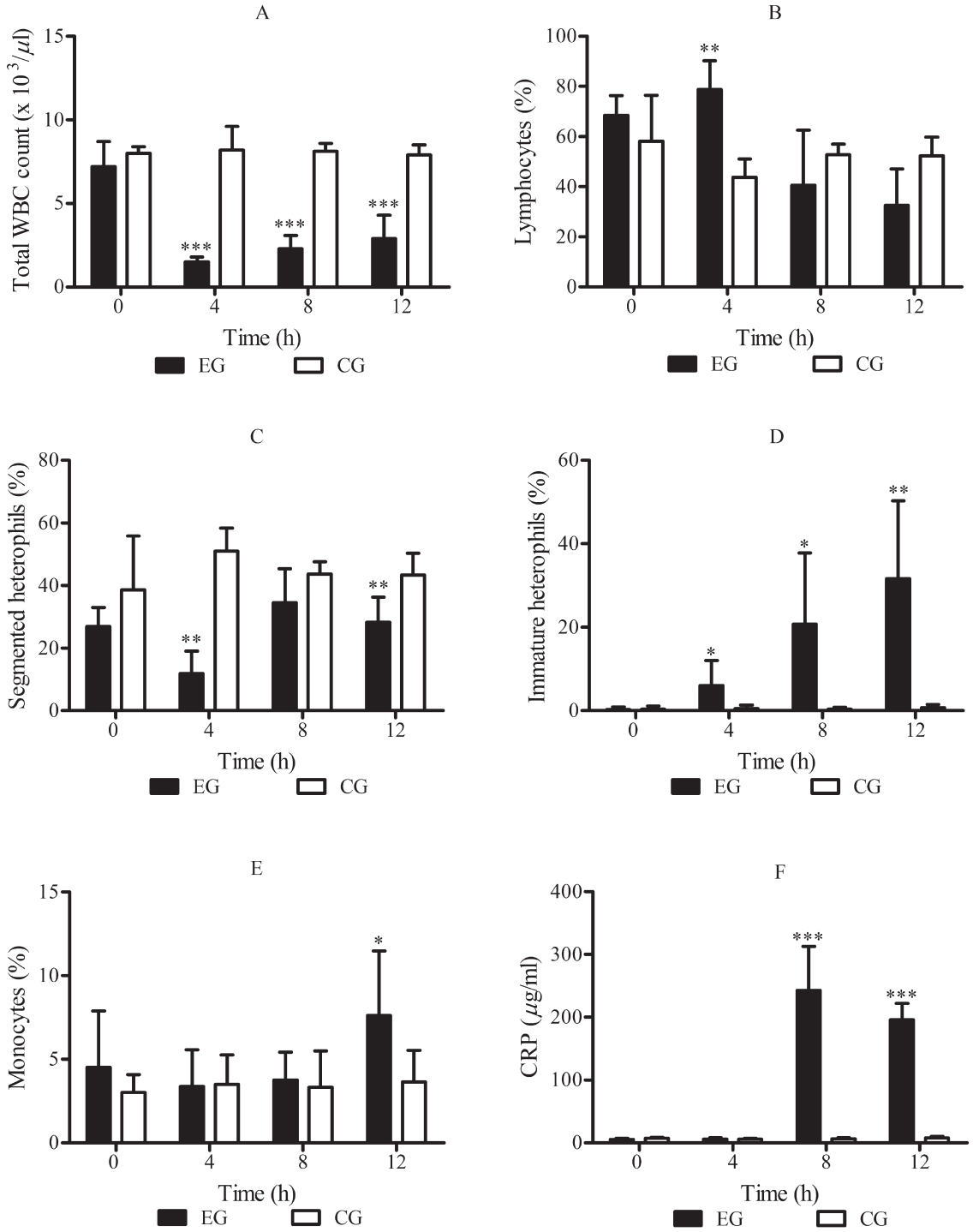

Fig. 1. Effects of experimental sepsis on the total WBC count (A), lymphocytes (B), segmented heterophils (C), immature heterophils (D), monocyte ratio (E), and serum CRP concentrations (F). EG, experimental group (n=8); CG, control group (n=6). *$P<0.05$; **$P<0.01$; ***$P<0.001$.

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heterophil ratio decreased at 4 h after operation, the immature heterophil ratio increased gradually starting at 4 h. No significant difference in serum CRP concentration was observed between EG and CG at 0 or 4 h. The highest serum concentration of CRP was 242.70 ± 70.43 μg/ml at 8 h.

The mean serum CKMB, AST, LDH, and cTnI concentrations are shown in Fig. 2. While the mean serum CKMB concentration did not change at different time points, the mean serum AST concentrations significantly increased (P<0.05) at 12 h. The peak serum LDH concentration was 581.75 ± 144.24 U/l at 4 h, and it remained high throughout the experiment. The mean serum cTnI concentrations in EG and CG before the experiment were 0.011 ± 0.003 and 0.018 ± 0.016 ng/ml, respectively. No changes in serum cTnI concentrations were observed in EG and CG at 0 or 4 h. In EG, the mean serum cTnI concentrations were 0.230 ± 0.209 and 1.177 ± 0.971 ng/ml at 8 and 12 h, respectively. In CG, the mean serum cTnI concentrations were 0.032 ± 0.014 and 0.031 ± 0.021 ng/ml at 8 and 12 h, respectively.

Macroscopic examination of the hearts after euthanasia revealed no pathological changes. Figure 3 shows the histopathological changes and cTnI immunoreactivity. Histopathologically, coagulated necrosis areas, mononuclear cell infiltrations, loss of muscle striations, picnotic nuclei, dark eosinophilic staining, and loss of cell boundaries were detected in EG (Figs. 3A and B). These changes were more extensive in the ventricular septum. Evaluation of the histopathological scores of all the rabbits revealed a significantly higher (P<0.001) degeneration/necrosis and cell infiltration scores in EG (2.62 ± 0.51) than in CG (0.62 ± 0.51). Moreover, cytoplasmic cTnI immunoreactivity was significantly reduced (P<0.01) in EG relative to that in CG (Figs. 3C and D). The percentage of cytoplasmic cTnI immunoreactivity was 52.5 ± 8.1% and 95.83 ± 3.86% in EG and CG, respectively.

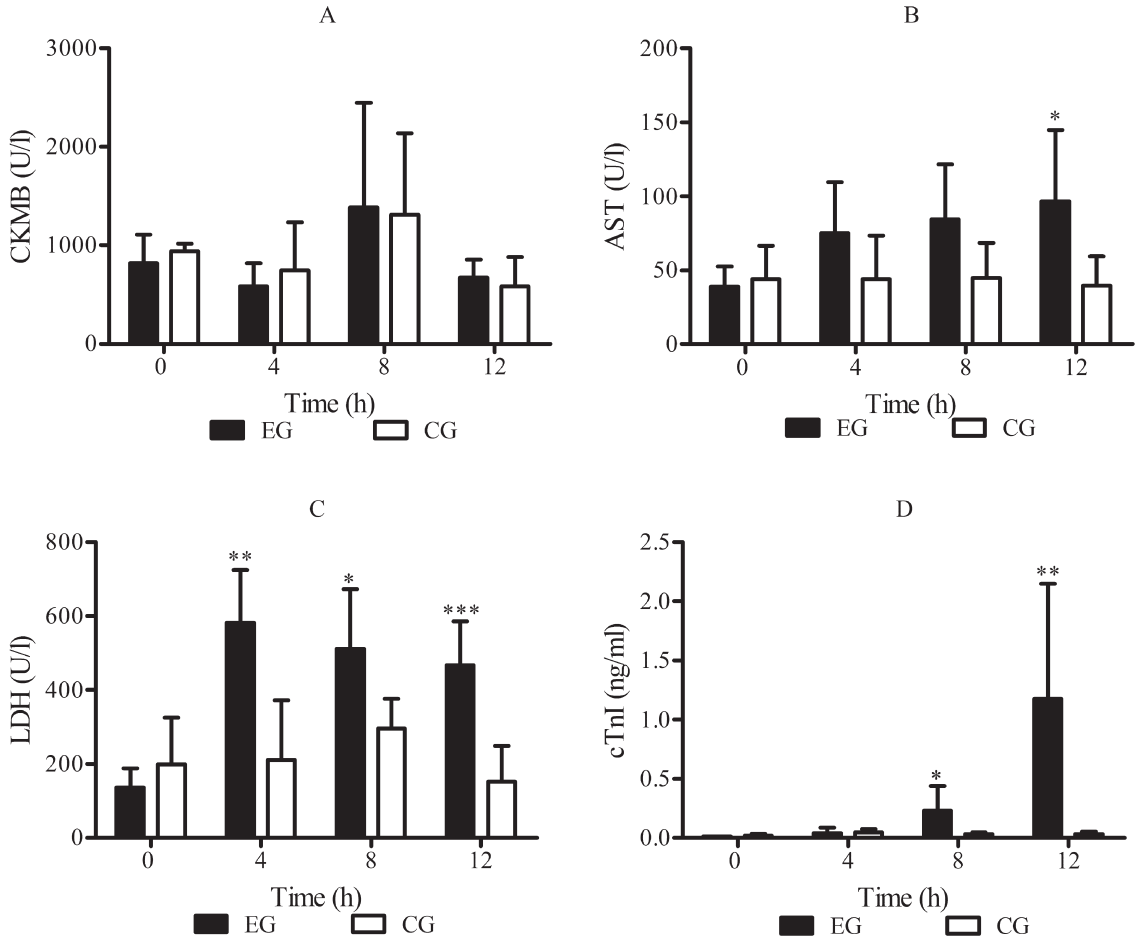

Fig. 2. Effects of experimental sepsis on serum creatine kinase isoenzyme MB (CKMB) (A), aspartate aminotransferase (AST) (B), lactate dehydrogenase (LDH) (C), and cardiac troponin I (cTnI) (D) concentrations. EG, experimental group (n=8); CG, control group (n=6). *P<0.05; **P<0.01; ***P<0.001.

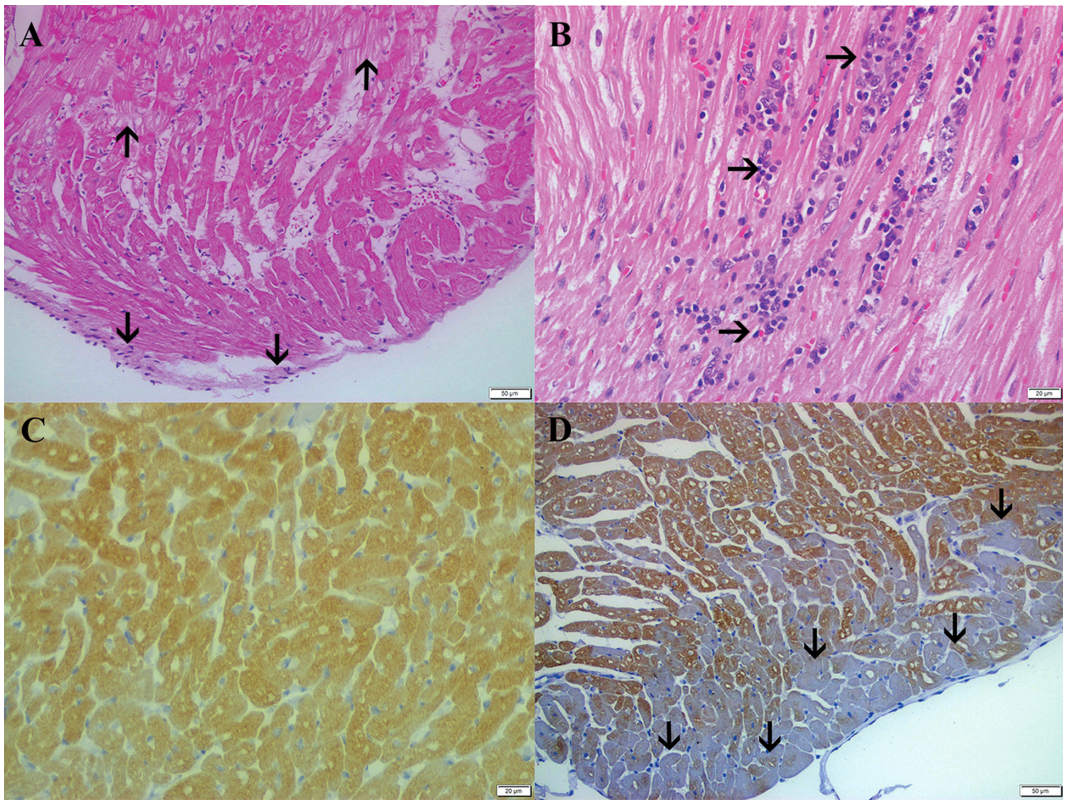

Fig. 3. A. Necrotic areas in a left ventricle section in an experimental group (EG) rabbit. H&E staining; bar=50 μm, (black arrow). B. Diffuse mononuclear cell infiltration in a right ventricle section in an EG rabbit. H&E staining; bar=20 μm, (black arrow). C. Normal cTnI immunoreactivity in a CG rabbit. ABC staining; bar=20 μm. D. Diffuse cytoplasmic cardiac troponin I (cTnI) loss in an EG rabbit. ABC staining; bar=50 μm, (black arrow).

Discussion

Our results showed that CLP in rabbits elicited a potent systemic inflammatory response, and it caused elevation of serum cTnI concentrations. Elevated concentrations of circulating cTnI have been reported in both naturally occurring and induced endotoxemia in both domestic and laboratory animals. An increased serum cTnI concentration was reported in dogs with systemic inflammatory response syndrome [16]. In a study on rabbit endotoxemia, serum cTnI concentrations increased after LPS infusion [21]. Serum cTnI concentrations in the present study also increased. In humans, cTnI concentrations begin to increase 4–12 h after acute myocardial infarction; the concentrations peaked at 12–48 h and remained high for 7–10 days [5, 15]. In an experimental endotoxemia study performed in calves, the serum cTnI concentration started to increase 3 h after LPS infusion, peaked at 6 h, and then gradually decreased until 24 h [29]. In another endotoxemia study performed in horses, the cTnI concentration peaked 1 h after LPS infusion [26]. In the present study, serum cTnI concentrations started to increase at 8 h post operation and peaked at 12 h. The difference between the reported results and the present results in terms of the time when cTnI concentration peaked is probably due to the early onset of systemic inflammatory response in LPS infusion [32].

The interaction among the circulating LPS, LPS-binding protein, and cluster of differentiation 14 activates the TLR4 signalling pathway; this activation results in secretion of proinflammatory cytokines, including TNF-α, IL-1β, and IL-6, and it causes a systemic inflammatory response [31, 41]. Although blood LPS concentrations have been reported in different experimental studies on sepsis, no experimental study has investigated the associations between blood LPS concentrations and clinical, haematological, and biochemical variables. In mice, serum LPS concentrations were 12.96 ± 1.82 EU /ml at 12 h after the CLP procedure [38]. In another study, plasma LPS concentrations increased from 0.28 ± 0.13 EU /ml to 2.27 ± 0.37 EU /ml at 10 h after low-dose endotoxin administration in rats [40]. In the present study, blood LPS concentrations were not measured. In the EG rabbits, the biochemical and pathological changes may have been associated with temporal changes in blood LPS concentrations. However, this is speculative and warrants further investigations using rabbit CLP models.

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CKMB, AST, and LDH have been used for many years to detect myocardial damage [22]. However, the use of these biomarkers is limited due to their low tissue specificity and sensitivity [2, 35]. In recent years, cTn has been used extensively in the diagnosis of myocardial damage because of its high tissue specificity and sensitivity [1, 27]. A study comparing the usefulness of LDH and CK isozymes with that of cTn revealed that LDH and CK isoenzymes were inefficacious in the presence of skeletal muscle injury, whereas cTn remained useful [28]. In the present study, no significant change in CKMB concentrations was observed at any time point. Although mean LDH and AST concentrations significantly increased at different time points, elevation of the concentrations of these enzymes may be associated with the effects of systemic inflammation in other tissues, such as those in the liver, intestines, skeletal muscle, and kidney [25].

In experimental endotoxemia studies, haematological changes are characterised by leukopenia and neutropenia followed by leukocytosis and neutrophilia, respectively [12]. In a rabbit endotoxemia study, while intravenous LPS infusion at a dose of 1 μg/kg resulted in leukopenia at 4 h post infusion, heterophilia occurred at 4 h post infusion [19]. In the present study, total WBC counts evidently decreased at 4 h in EG. Additionally, heteropenia was present at 4 h. In our study, heteropenia was most likely caused by intense influx of heterophils from the circulation to the inflamed tissues [4]. Besides the total WBC count and differential leukocyte ratio, C-reactive protein is accepted as a major acute phase reactant in the presence of inflammatory stimuli in rabbits [7]. The results of an experimental inflammation study involving rabbits demonstrated that while plasma CRP concentrations were lower than 10 μg/ml in control rabbits, CRP concentrations started to increase at 6 h after turpentine injection and peaked at 514 μg/ml at 36 h [14]. In another study involving rabbits, after three intravenous injection of LPS at 6-h intervals, the mean serum CRP concentration was 81.4 ± 3.3 μg/ml at 24 h [30]. In our study, the peak serum CRP concentration was 242.70 ± 70.43 μg/ml at 8 h. In EG, the mean serum CRP concentration initially increased at 8 h. Although serum CRP concentration changes were measured to follow the systemic inflammatory response in the present study, serum LDH concentrations quickly increased before serum CRP concentrations increased. LDH is a cytoplasmic enzyme that has wide tissue distribution, including the tissues of the heart, skeletal muscle, kidney, intestines, liver, lung, pancreas, and red blood cells [23, 24]. In the present study, because total LDH activity was measured but not the activities of LDH isoenzymes and other tissue-specific enzymes, such as alanine aminotransferase, CK, creatinine, and blood urea nitrogen, the exact cause of the early increase in LDH activity was difficult to predict. This early increase in LDH may be associated with skeletal muscle damage, which may have occurred while restraining the rabbits for blood collection, or may be associated with invisible haemolysis in serum samples [20, 24, 42].

In EG, the rabbits showed a significant increase in serum cTnI concentrations compared with the rabbits in CG. When a damage that disrupts membrane integrity in cardiac myocytes occurs, cardiac troponins are released into the circulation [39]. The correlations among the circulating cTnI concentration, severity of histopathological changes, and loss of cTnI immunoreactivity have been demonstrated in various studies [6, 18, 37]. In the present study, loss of cTnI immunoreactivity and severe histopathological changes characterised by coagulated necrosis areas, mononuclear cell infiltration, loss of muscle striation, picnotic nuclei, dark eosinophilic staining, and loss of cell boundries in EG rabbits were observed; however, no correlation was detected between histopathological changes and serum cTnI concentrations or between cTnI immunoreactivity and serum cTnI concentrations at the end of the experiments. Of the eight rabbits in EG, three rabbits showed increased serum cTnI concentrations, but histopathological lesion scores were mild, and loss of cytoplasmic cTnI immunoreactivity was not severe. It is possible that focal lesions were not sampled in the examined sections.

In conclusion, the results of this study demonstrated that CLP induced both haematological and biochemical changes characterised by leukopenia, heteropenia, and elevated serum concentrations of CRP, cTnI, AST, and LDH. Additionally histopathological changes and loss of cTnI immunoreactivity in the cardiac tissue confirmed cardiac muscle damage in rabbit CLP model. Further studies are required to determine whether or not myocardial damage is reversible in the rabbit CLP model.

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