ORIGINAL ARTICLE

CONTENTS

Ghrelin attenuates the inflammatory response induced by experimental endotoxemia in mice

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ABSTRACT

Aim: The aim of this research is to assess the anti-inflammatory effect of ghrelin in mice models of polymicrobial sepsis.

Materials and Methods: 35 male albino Swiss mice, ages 8-12 weeks, weighing 23-33g, were randomly separated into five groups n = 7; normal group was fed their usual diets until time of sampling, the sham group subjected to Anaesthesia and laparotomy, sepsis group subjected to cecal ligation and puncture, vehicle group was given an equivalent volume of intraperitoneal saline injections immediately after cecal ligation and puncture, and the ghrelin group was treated with 80 µg/kg of ghrelin intraperitoneal injections immediately following cecal ligation and puncture. Twenty hours after cecal ligation and puncture, mice were sacrificed; myocardial tissue and serum samples were collected. Serum IL-1β, NF-κB, and TLR4 levels were measured, and inflammatory response's effects on cardiac tissue were evaluated.

Results: The mean serum IL-1β, NF-κB, and TLR4 levels were markedly elevated in the sepsis and vehicle groups than in the normal and sham groups. The mean serum levels of IL-1β, NF-κB, and TLR4 were considerably lower in the ghrelin-treated group than in the vehicle and sepsis groups. Myocardium tissue of the normal and sham groups showed normal architecture. The sepsis and vehicle groups had a severe myocardial injury. The histological characteristics of ghrelin-treated mice differed slightly from those of the normal and sham groups.

Conclusions: Our study concluded that ghrelin exerts anti-inflammatory effects in polymicrobial sepsis, as indicated by a considerable decrease in the IL-1β, NF-κB and TLR4 serum levels.

KEY WORD: Ghrelin, NF-κB, CLP, IL-1β, sepsis, TLR4

Wiad Lek. 2024;77(4):652-658. doi: 10.36740/WLek202404106 DOI

INTRODUCTION

Sepsis is an unregulated systemic inflammatory reaction, which could lead to death from multiple organ failure. The fundamental cause of a poor prognosis in sepsis is an imbalance between the pro- and anti-inflammatory responses. About 31 million individuals worldwide suffer from sepsis yearly, leading to more than 5 million deaths. Due to the lack of data from numerous low-income areas, these results likely underestimate the true worldwide impact of sepsis [1]. In sepsis, signal transduction is triggered by the binding of damage-associated molecular patterns (DAMPs) derived from injured tissues or pathogen-associated molecular patterns (PAMPs) derived from microbes to toll-like receptors (TLRs) on monocytes and antigen-presenting cells [2], resulting in the migration of nuclear factor kappa B (NF-κB) from the cytoplasm into the nucleus of the cell. After that, NF-κB stimulates the synthesis of proinflammatory cytokines such as interferon, interleukin (IL)-1, IL-18, and tumour necrosis factor-alpha (TNF-α) [3]. Even though there is an increase in knowledge about the pathophysiology of sepsis, therapeutic control of sepsis has advanced slowly.

Ghrelin is a polypeptide hormone comprising 28 amino acids, primarily released in the stomach [4]. It is responsible for increasing both the release of growth hormone (GH) and the appetite. Numerous studies demonstrated that some mammalian peptide hormones have promising effects against sepsis, including vasopressin, oxytocin, human chorionic gonadotropin, ghrelin, and glucagon [5]. The hormone ghrelin has anti-inflammatory effects in disorders affecting many body systems, including the endocrine, immune, digestive, skeletal, respiratory, metabolic, and central nervous systems. Treatment with ghrelin reduces inflammation, which in turn reduces the severity of many conditions such as inflammatory bowel disease, arthritis, sepsis, diabetic nephropathy, obesity, pancreatitis, cachexia, and some rodent models of chronic inflammation [6-9]. Ghrelin suppresses proinflammatory cytokine expres-

sion by macrophages, T lymphocytes, and monocytes [10]. Numerous investigations have verified that ghrelin has an immunomodulatory effect on sepsis. Ghrelin prevents lipopolysaccharide (LPS) stimulated microglia from releasing inflammatory cytokines [11], moreover, it inhibits IL-6 release by LPS-stimulated dopaminergic nerve cells [12]. Exogenous ghrelin significantly reduce LPS-induced synthesis of proinflammatory cytokines in mice [13]. Even 12–24 hours after cecal ligation and puncture (CLP), ghrelin reduced mortality and markedly lowered both pathological scores and clinical parameters of sepsis in mice. IL-1β is a major proinflammatory cytokine responsible for the modulation of hosts' innate immune system response [14]. IL-1β is a cardio-depressant proinflammatory mediator that increases markedly in both human and animal models during sepsis. TLR4 is a unique pattern recognition receptor expressed on the surface of immune cells [15, 16]. TLR4 stimulates the host immunological response towards bacterial, fungal, viral, and malaria infections. NF-κB is a rapid-acting transcription factor that modulates diverse cellular processes and is involved in septic shock syndrome, chronic inflammatory conditions, multiple organ dysfunction, and viral infections. NF-κB augments the synthesis of proinflammatory cytokines, such as TNF-α, IL-12, and IL-1β [17-20].

AIM

The aim of this research is to assess the anti-inflammatory effect of ghrelin in mice models of polymicrobial sepsis.

MATERIALS AND METHODS

ANIMALS PREPARATION

Thirty-five male albino Swiss mice were acquired from the Iraqi Center for Cancer Research. They were maintained at the Faculty of Pharmacy/University of Kufa animal house. They were housed in cages with a 12-hour light/12-hour dark cycle, temperatures ranging from 22-24°C, humidity levels ranging from 60-65%, and unrestricted access to water and food. The study was conducted in the Laboratory of Clinical laboratory department/Faculty of Pharmacy, University of Kufa from December 5, 2022, until February 25, 2023.

STUDY DESIGN

The mice were acclimatized for a week and then randomly divided into five groups of 7 animals each:

- Normal group: Mice were fed their usual diets until the time of sampling.
- Sham group: Mice were subjected to Anaesthesia and laparotomy; the sham group was the negative surgical control group.
- Sepsis group: Mice were subjected to the CLP procedure; it was the positive surgical control group.
- Vehicle group: An equivalent volume of normal saline intraperitoneal injections was given immediately following CLP.
- Ghrelin-treated group: Mice were treated with 80 µg/kg of recombinant human ghrelin intraperitoneal injections immediately following CLP.

Twenty hours after CLP, mice were sacrificed; myocardial tissue and serum samples were collected.

EXPERIMENTAL MODEL OF SEPSIS

According to recent research, this study chose mice to induce polymicrobial sepsis using the CLP model [14]. Briefly, 0.01 mg/g xylazine and 0.1 mg/g ketamine were injected intraperitoneally to mice to induce anesthesia. [21-26]. An abdominal midline incision of 1.5 cm was made. The cecum was ligated beneath the Bauhin valve, double perforated with a 22-gauge needle, and slightly squeezed to force a small stool out of the puncture. Then, the cecum returned to its anatomical position, and a 4-3 surgical suture was used to close the abdominal incisions.

PREPARATION OF GHRELIN

Recombinant pure human ghrelin 95% (Elabscience, USA) was dissolved in normal saline. Then, 80µg\kg ghrelin was administered intraperitoneally immediately after CLP.

SAMPLES COLLECTION

BLOOD SAMPLES

The blood samples were gathered by heart puncture before sacrificing mice. It was placed in a gel tube and left at room temperature for 1 hour. Serum was separated by centrifuging blood for 20 minutes at 4000 rpm. The enzyme-linked immunosorbent assay (ELISA) technique measured serum IL-1β, NF-κB, and TLR4 levels.

TISSUE SAMPLES

The cardiac tissue was fixed in a 10% formaldehyde solution for 20 hours. After dehydration and clearing, cardiac tissue was embedded in a paraffin block, and a 5 µm thick section was sliced using a microtome. Hematoxylin and eosin were employed to stain the tissue slices before being examined under a light microscope.

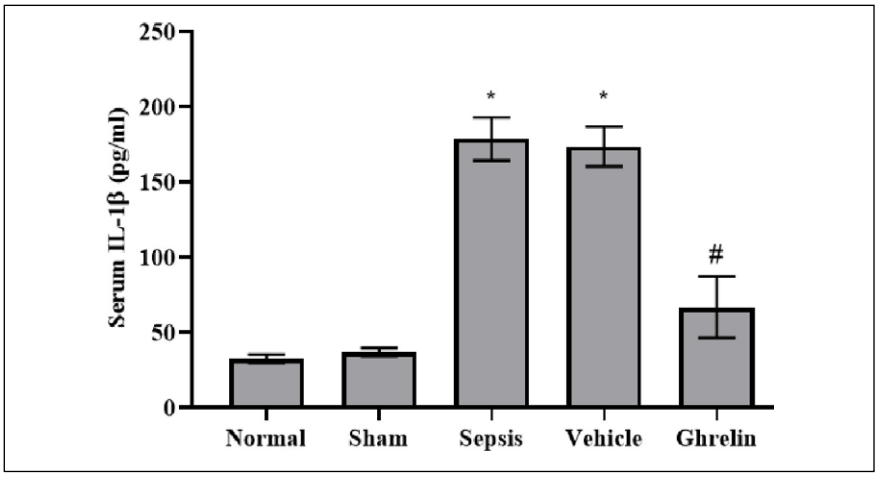

Fig. 1. Serum IL-1β level in the experimental groups: * significant, p<0.001 *vs*. normal or sham groups; # significant, p<0.001 *vs*. sepsis or vehicle groups.

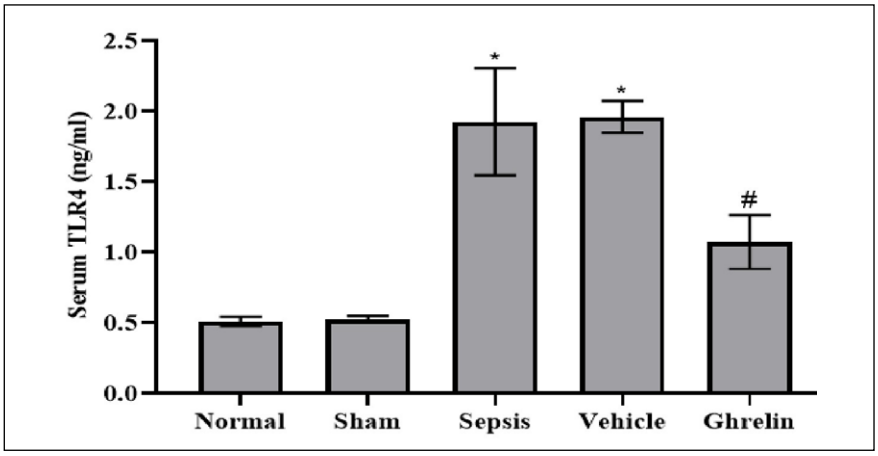

Fig. 2. Serum TLR4 level in the experimental groups: * significant, p<0.001 *vs*. normal or sham groups; # significant, p<0.001 *vs*. sepsis or vehicle groups.

HISTOLOGICAL EXAMINATION

The extent of cardiac damage was evaluated for each cardiac section using an optical microscope, and photographs of the sections were obtained. Histological sections of the heart were scored according to the Zingarelli protocol (36). The criteria for this scoring system were:

- Score 0: There is no damage.
- Score 1: Localized necrosis with interstitial oedema.
- Score 2: Diffused swelling of the cardiomyocytes.
- Score 3: Leukocyte infiltration and contraction band.
- Score 4: Contraction band, neutrophil infiltration, and haemorrhage.

STATISTICAL ANALYSIS

Version 8.1 of GraphPad Prism was employed to conduct the statistical analysis. Mean ± standard error mean (SEM) was used to display the data, and all groups were compared by one-way the analysis of variance (ANOVA) test. The Bonferroni method for multiple comparisons was subsequently employed to conduct post-hoc tests. Histopathological changes were compared between groups by a non-parametric test followed by Dunn's post hoc test. All analyses were considered statistically significant if $P < 0.05$.

RESULTS

EFFECT OF GHRELIN TREATMENT ON IL-1B AFTER POLYMICROBIAL SEPSIS

Serum IL-1β levels were markedly elevated in the sepsis group than in the normal and sham groups (p<0.001). There were no statistically significant variations in serum IL-1β levels between the vehicle and sepsis groups or between the normal and sham groups. Compared to the sepsis and vehicle groups, ghrelin administered immediately after the CLP procedure considerably lowered serum IL-1β levels (p<0.001) (Fig. 1.).

EFFECT OF GHRELIN TREATMENT ON TLR4 AFTER POLYMICROBIAL SEPSIS

Serum TLR4 levels were markedly elevated in the sepsis group than in the normal and sham groups (p<0.001). There were no statistically significant variations in serum TLR4 levels between the vehicle and sepsis groups or between the normal and sham groups. Compared to the sepsis and vehicle groups, ghrelin administered immediately after the CLP procedure considerably lowered serum TLR4 levels (p<0.001) (Fig.2).

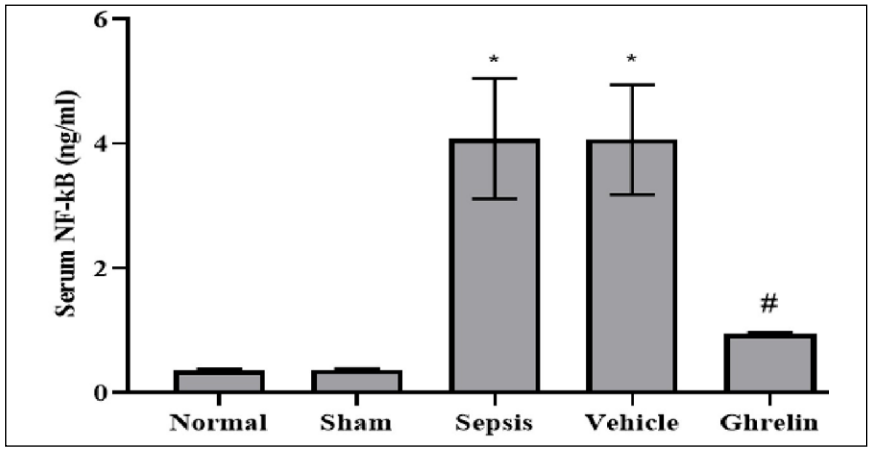

Fig. 3. Serum NF-kB level in the experimental groups: * significant, p<0.001 *vs*. normal or sham groups; # significant, p<0.001 *vs*. sepsis or vehicle groups.

EFFECT OF GHRELIN TREATMENT ON NF-KB AFTER POLYMICROBIAL SEPSIS

Serum NF-κB levels were markedly elevated in the sepsis group than in the normal and sham groups (p<0.001) (Fig.3).

There were no statistically significant variations in serum NF-κB levels between the vehicle and sepsis groups or between the normal and sham groups. Compared to the sepsis and vehicle groups, ghrelin administered immediately after the CLP procedure considerably lowered serum NF-κB levels (p<0.001) (Fig.3).

HISTOPATHOLOGICAL CHANGES OF MYOCARDIAL TISSUE AFTER POLYMICROBIAL SEPSIS

Myocardium tissue of the normal and sham groups showed normal architecture with distinct myocyte boundaries and without erythrocyte leakage and leukocyte infiltration (Fig.4. A-B).

All mice in these groups have normal histopathological findings (score 0), as shown in (Fig.5). The sepsis and vehicle groups had a highly severe myocardial injury (score 4), characterized by the appearance of contraction bands, interstitial oedema, leukocyte infiltration, and erythrocyte extravasation (Fig.4 C-D). The mean histological score was significantly higher in the vehicle and sepsis groups than in the normal and sham groups (p<0.001) (Fig.5). The ghrelin-treated group had a mild myocardial injury (score 1) (Fig.4 E). The mean histological score was markedly lower in the ghrelin-treated group than in the sepsis and vehicle groups (p<0.01) (Fig.5).

DISCUSSION

Sepsis is an unregulated systemic inflammatory reaction that could lead to death from multiple organ failure. This study found significantly higher serum levels of IL-1β in vehicle and sepsis groups compared to the normal and sham groups. In contrast, the ghrelin-treated group had markedly lower serum IL-1β levels than the vehicle and sepsis groups. Yousif et al. (2020) [27] found that IL-1β levels in heart tissue and plasma were elevated in septic mice, which was related to reduce cardiac contractility and induced myocardial damage. Similarly, Zigam et al. (2023) [22] demonstrated that serum IL-1β levels increased significantly 24 hours after CLP surgery compared to the sham group. In contrast, Corrêa da Silva et al. (2019) [28] showed that ghrelin has an immunoregulatory effect on LPS-activated macrophage, as demonstrated by stimulation of IL-12 expression and suppression of IL-1β release. Moreover, Shao et al. (2020) [29] found that ghrelin injection decreased the synthesis of IL-1β in the lung tissues in a mouse model of acute lung injury. Qiu et al. (2022) [30] showed that ghrelin administration had a considerable suppressive action on the expression of IL-1β compared to the rat model of subarachnoid haemorrhage. According to our results, it is evident that the studies mentioned above all support the results we obtained. This study found a significantly higher serum level of TLR4 in sepsis and vehicle groups compared to the normal and sham groups. By contrast, the ghrelin-treated group had markedly lower serum levels of TLR4 compared to the vehicle and sepsis groups. According to Wang et al. (2019), CLP-induced sepsis stimulates the NF-κB phosphorylation and TLR4 activation in the lung tissue of rats. In contrast, Sun et al. (2016) [24] demonstrated that ghrelin inhibited the protein expression of TLR4 in mice after myocardial ischemia/reperfusion injury. Wang et al. (2019) revealed that the ability of ghrelin to reduce apoptosis and inflammation could be related to the inhibition of TLR4/NF-κB in the testes of mice subjected to immobilization stress. Liu et al. (2023) show that the TLR4 pathway was activated in response

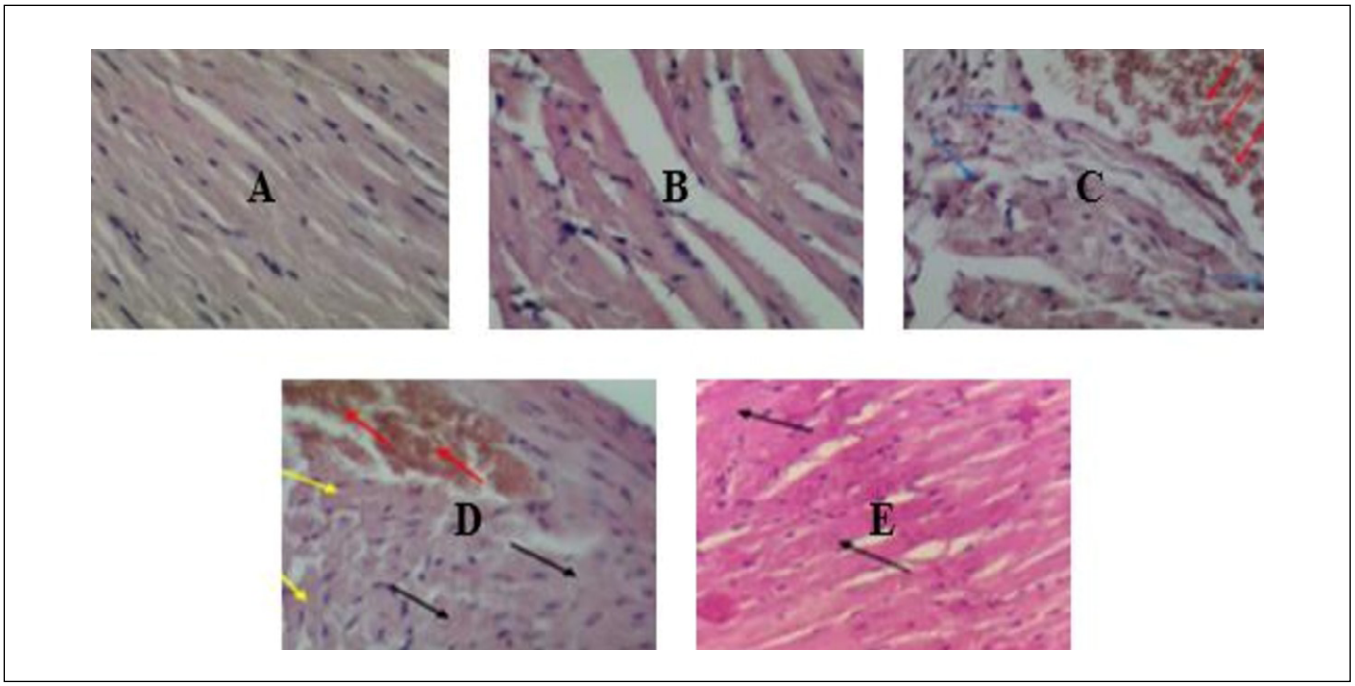

Fig. 4. Photograph of the heart section stained with H&E (X400). A: normal group showed normal architecture (score 0); B: sham group showed normal architecture (score 0); C: sepsis group, showed haemorrhage (red arrows) and leukocyte infiltration (blue arrows); D: vehicle group showed haemorrhage (red arrows), cell swelling (yellow arrows), and necrosis (black arrows); E: ghrelin group showed mild necrosis (black arrows).

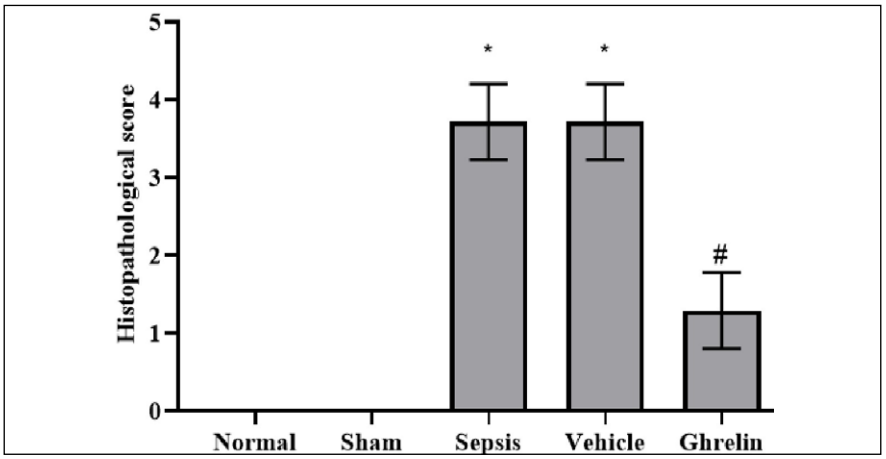

Fig. 5. Histopathological score in the experimental groups: * significant, $p<0.001$ *vs*. normal or sham groups; # significant, $p<0.01$ *vs*. sepsis or vehicle groups.

to high glucose or hyperglycemia and significantly suppressed by ghrelin administration in vitro and in vivo. The current study revealed a significantly higher serum NF-κB level in sepsis and vehicle groups compared to the normal and sham groups. However, the serum NF-κB level decreased substantially in the ghrelin-treated group compared to the sepsis and vehicle groups. Yousif et al. (2020) [27] found that activation of NF-κB and phosphorylation of mitogen-activated protein kinase (MAPK) are increased in septic mice and cause a decrease in left ventricle function. In addition, it was reported that MAPK/NF-κB pathway activation is associated with increased plasma TNF-α, IL-6, and IL-1β levels, causing a further decrease in left ventricle function. Yildiz et al. (2021) revealed that the NF-κB expression was substantially increased in the lung tissue of CLP rats. While Liu et al. (2019) demonstrated that ghrelin administration significantly reduced NF-κB expression in rats' autoimmune encephalomyelitis models, suggesting its Neuroprotective effects. Qu et al. (2019) demonstrated that ghrelin treatment reduces NF-κB activation in mouse models of psoriasis. Moreover, Zheng et al. (2017) [14] showed that ghrelin inhibited the translocation of NF-κB in alveolar macrophages obtained from septic rats. Song et al. (2021) reported that ghrelin suppresses inflammation and autophagy associated with chronic obstructive pulmonary disease by blocking the NF-κB signalling pathways. In polymicrobial sepsis, oxidative stress and excessive production of proinflammatory cytokines result in myocardial histological abnormalities. These mediators cause cardiac tissue necrosis, pycnosis, karyolysis, and karyorrhexis.

This study showed that vehicle and sepsis groups had substantially higher cardiac tissue injury than normal and sham groups. The histopathological damage scores were mostly highly severe (score 4) for sepsis and vehicle groups. While ghrelin administration significantly reduces cardiac tissue injury compared to sepsis and vehicle groups. The ghrelin-treated group had mild (score1) histopathological damage scores. Topcu et al. (2022) indicated that elevated proinflammatory cytokine and oxidative stress in septic cardiac tissue result in severe injury manifested as degenerative cardiomyocytes, vascular congestion, and oedema. However, Topcu et al. (2022) demonstrated that ghrelin could protect rats against septic-induced cardiotoxicity by reducing the inflammatory response and apoptosis. A recent study showed that ghrelin ameliorates thyroxin-induced myocardial injury in rats through anti-inflammatory effects, antioxidant effects, and decreased expression of heart renin-angiotensin system components.

CONCLUSIONS

Our study concluded that ghrelin exerts anti-inflammatory effects in polymicrobial sepsis, as indicated by a considerable decrease in proinflammatory cytokines levels, including IL-1β, NF-κB and TLR4.

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CONFLICT OF INTEREST

The Authors declare no conflict of interest

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RECEIVED: 15.07.2023
ACCEPTED: 05.04.2024