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Ghrelin inhibits inflammatory response and apoptosis of myocardial injury in septic rats through JAK/STAT signaling pathway

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Abstract. – OBJECTIVE: The purpose of this study was to explore the influence of Ghrelin on myocardial injury of septic rats through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway.

MATERIALS AND METHODS: A total of 36 Sprague-Dawley rats were randomly divided into normal group (n=12), model group (n=12), and Ghrelin group (n=12). The rats in the normal group were fed normally, while those in the model group were intraperitoneally injected with endotoxin to establish the sepsis model. The rats in the Ghrelin group were given intraperitoneal injection of Ghrelin solution to prepare the sepsis model. 9 h later, the specimens were obtained. Then, the expressions of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) were detected via immunohistochemistry, and the protein expressions of phosphorylated JAK (p-JAK) and STAT3 were determined by Western blotting (WB). Next, enzyme-linked immunosorbent assay (ELISA) was performed to measure the content of IL-6 and TNF-α, and quantitative Polymerase Chain Reaction (qPCR) was applied to examine the messenger ribonucleic acid (mRNA) expressions of JAK and STAT3. Finally, the cell apoptosis was detected through terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay.

RESULTS: The results of immunohistochemistry showed that compared with those in the normal group, the positive expression levels of IL-6 and TNF-α were markedly increased in other groups ($p<0.05$), while in comparison with those in the model group, the positive expression levels of IL-6 and TNF-α were decreased significantly in the Ghrelin group ($p<0.05$). The WB results indicated that the model group and Ghrelin group had remarkably higher protein expression levels of p-JAK and STAT3 than the normal group ($p<0.05$), and Ghrelin group exhibited notably lower protein expression levels of p-JAK and STAT3 than the model group ($p<0.05$). According to the results of qPCR, the relative mRNA expression levels of JAK and STAT3 were distinctly raised in the model group and Ghrelin group in comparison with those in the normal group ($p<0.05$), while they were reduced evidently in the Ghrelin group compared with those in the model group ($p<0.05$). Furthermore, it was manifested in the results of ELISA that the model group and Ghrelin group had prominently elevated content of TNF-α and IL-6 compared with normal group ($p<0.05$), and Ghrelin group displayed significantly lowered content of TNF-α and IL-6 in comparison with the model group ($p<0.05$). Moreover, the TUNEL results revealed that the apoptosis rate was remarkably higher in the other two groups than that in the normal group ($p<0.05$), while it was evidently lower in the Ghrelin group than that in the model group ($p<0.05$).

CONCLUSIONS: Ghrelin can inhibit inflammatory response and apoptosis in the process of myocardial injury in septic rats by repressing the JAK/STAT signaling pathway.

Key Words:
Sepsis, Inflammation, Ghrelin, JAK/STAT signaling pathway.

Introduction

Sepsis, a kind of critical disease induced by infection, is characterized by systemic inflammatory response syndrome, multiple organ failure, shock, etc.[1,2]. It often leads to multiple organ injury all over the body, such as lung injury, heart injury, kidney damage and liver damage, and can cause death of the patients, seriously threatening the life and health of the patients[3,4]. Therefore, sepsis has attracted intensive attention of clinical researchers around the world due to its relatively high morbidity and mortality rates.

Inflammation is an important pathological damage of sepsis. In particular, systemic inflammation poses very severe damage to the body[5,6].

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The sepsis-induced systemic inflammation, especially the heart inflammation, usually leads to secondary inflammation in multiple organs in organisms, resulting in such life-threatening diseases as myocarditis in the patients. The Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway, a vital cellular signal transduction pathway, plays critical regulatory roles in various physiological and pathological responses, including inflammation[7]. Particularly, the JAK/STAT signaling pathway participates in modulating the onset and development of sepsis by regulating inflammation. Kacimi et al[8] demonstrated that the activated JAK and STAT3 in the JAK/STAT signaling pathway control the expressions of the tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) to aggravate inflammation, thus triggering secondary pathological responses of sepsis.

As an important type of brain-gut polypeptide, Ghrelin has favorable inhibitory effects on inflammation, but the mechanism of its inhibition on inflammation remains unclear. Therefore, the purpose of the present study was to clarify the relevant mechanism of Ghrelin in treating sepsis and to investigate whether Ghrelin can exert good therapeutic effects on myocardial injury of septic rats by suppressing inflammation through the regulation of the JAK/STAT signaling pathway.

Materials and Methods

Laboratory Animals and Grouping

A total of 36 Sprague-Dawley rats weighing (18±2) g (half males and half females) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All the rats were divided into normal group (n=12), model group (n=12), and Ghrelin group (n=12) using a random number table. This study was approved by the Animal Ethics Committee of The Second Military Medical University Animal Center.

Experimental Reagents and Instruments

Lipopolysaccharide (Boster, Wuhan, China), primary antibodies: anti-STAT3 antibody (Abcam, Cambridge, MA, USA) and anti-JAK antibody (Abcam, Cambridge, MA, USA), immunohistochemistry kit (Vazyme, Nanjing, China), terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) apoptosis kit and ribonucleic acid (RNA) extraction kit (Vazyme, Nanjing, China), reverse transcription kit (Vazyme, Nanjing, China), enzyme-linked immunosorbent assay (ELISA) kit (Sigma, St. Louis, MO, USA), light microscope (Leica, Wetzlar, Germany), fluorescence quantitative Polymerase Chain Reaction (qPCR) instrument (ABI, Applied Biosystems, Foster City, CA, USA).

Model Establishment

The rats were intraperitoneally injected with 7% chloral hydrate (5 mL/kg). After successful anesthesia, lipopolysaccharide was intraperitoneally injected into the rats (6 mg/kg), so as to establish a rat model of sepsis.

Treatment in Each Group

The rats in the normal group were raised normally, without any treatment. The rats in the model group were given intraperitoneal injection of normal saline once a day after sepsis modeling. In the Ghrelin group, the rats received intraperitoneal injection of Ghrelin solution (200 μg/kg) first, and the sepsis model was prepared 30 min later. The specimens were obtained at 9 h after modeling.

Specimen Acquisition

After successful anesthesia, cardiac tissues of 6 rats in every group were fixed with paraformaldehyde, and then, the paraffin-embedded tissue sections were prepared for immunohistochemistry and TUNEL assay. Thereafter, the cardiac tissue specimens were acquired directly from the remaining 6 rats in each group, preserved, and used for Western blotting (WB) and qPCR assays.

Immunohistochemistry

The tissues embedded in paraffin in advance were prepared into 5 μm-thick sections, spread in warm water at 42°C, collected, and baked, so as to prepare the paraffin-embedded tissue sections. Then, the sections obtained were soaked in the xylene solution and gradient alcohol for routine deparaffinization until rehydration. Subsequently, the above sections were immersed in citric acid buffer solution and heated repeatedly in a microwave oven for 3 times (heating for 3 min and braising for 5 min every time), so as to realize sufficient antigen retrieval. After rinsing, endogenous peroxidase blocker was added dropwise onto the specimens and reacted for 10 min. Next, the specimens were rinsed and added with goat serum for sealing for 20 min, and anti-IL-6 primary antibody (1:200) and anti-TNF-α primary antibody (1:200) were added after the goat serum blocking buffer was shaken off, followed by cul-

ture in a refrigerator at 4°C overnight. The next day, the specimens were rinsed, added with the secondary antibody solution, and reacted for 10 min. After rinsing adequately, streptavidin-peroxidase solution was added for reaction for 10 min, followed by color development with 3,3-diaminobenzidine (DAB) (Solarbio, Beijing, China) in drops, counterstaining of the nucleus with hematoxylin, mounting, and observation.

WB Assay

The lysis buffer was added into the cryopreserved cardiac tissues for 1 h of ice bath, then, the tissues were put into a centrifuge for centrifugation at 14,000 g for 10 min, and the proteins were quantified using bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Next, the absorbance and standard curve of the proteins were obtained through a microplate reader, which were applied to calculate the protein concentration in tissues. Subsequently, the proteins in tissue specimens were subjected to denaturation and separation *via* sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The position of the Marker proteins was observed, and the electrophoresis was stopped when the Marker proteins reached the bottom of glass plate in a straight line. Later, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland) and reacted with blocking buffer for 1.5 h. After that, anti-phosphorylated JAK (p-JAK) primary antibody (1:1000), anti-STAT3 primary antibody (1:1000), and secondary antibody (1:1000) were added in sequence. Finally, the image was fully developed with chemiluminescent reagent in the dark for 1 min after rinsing.

ELISA

The cardiac tissues stored for use were ground. According to the instructions of the ELISA kit, the specimens were loaded, the standard substance, biotinylated antibody working solution, and enzyme conjugate working solution were added, and the plate was washed successively. Finally, the tissues were placed in the microplate reader and detected at 450 nm.

QPCR assay

The total RNA was firstly extracted and then reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using the reverse transcription kit, with a reaction system of 20 μL. Reaction conditions: reaction at 51°C for 2 min, pre-denaturation at 96°C for 10 min, denaturation at 96°C for 10 s, and annealing at 60°C for 30 s, 40 cycles in total. The relative expression of relevant messenger RNA (mRNA) was calculated with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. The detailed primer sequences are shown in Table I.

Detection of Cell Apoptosis Via TUNNEL Assay

The tissues embedded in paraffin in advance were made into 5 μm-thick sections following spreading in warm water at 42°C, collection, and baking. Then, the sections obtained were soaked in xylene solution and gradient alcohol for routine deparaffinization until rehydration. TdT solution was added in drops for reaction in the dark for 1 h, and deionized water was added dropwise and incubated for 15 min to terminate the reaction. After that, hydrogen peroxide was added in drops to block the activity of endogenous peroxidase, followed by dropwise addition of working solution for reaction for 1 h. Finally, after rinsing, DAB solution was added for color development, followed by rinsing, mounting, and observation.

Statistical Analysis

In this research, Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp., Armonk, NY, USA) was adopted for statistical analysis. The enumeration data were expressed as mean ± standard deviation. *t*-test was performed for data meeting normal distribution and homoge-

Table I. Primer sequences.

Name	Primer sequence
JAK	Forward primer: 5' TCCACCAAGAAGCTGAGCGAG 3' Reverse primer: 5' GTCCAGCCCATGATGGTTCT 3'
STAT3	Forward primer: 5' TTCTTTGAGTTCGGTGGGGTC 3' Forward primer: 5' TGCATATTTGTTTGGGGCAGG 3'
GAPDH	Forward primer: 5' ACGGCAAGTTCAACGGCACAG 3' Reverse primer: 5' GAAGACGCCAGTAGACTCCACGAC 3'

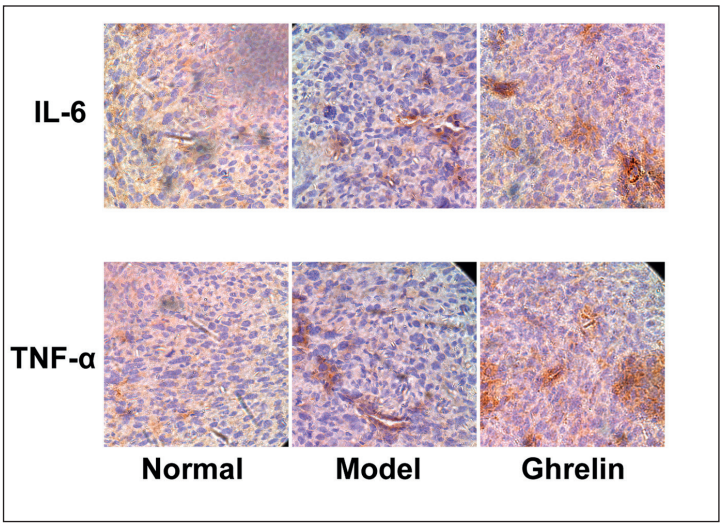

Figure 1. Immunohistochemistry (magnification: 200×).

neity of variance, corrected *t*-test for those meeting normal distribution and heterogeneity of variance, and non-parametric test for those not meeting normal distribution and homogeneity of variance. The ranked data were subjected to rank sum test, and the enumeration data underwent chi-square test. *p*-values < 0.05 were considered statistically significant

Results

Immunohistochemistry

As shown in Figure 1, the tissues with positive expression were sepia. There were lower positive expressions of IL-6 and TNF-α in normal group and higher positive expressions in other groups. The statistical results manifested that the average optical densities of IL-6 and TNF-α positive expressions rose significantly in the model group and Ghrelin group in comparison with that in the normal group, with statistically significant differences ($p<0.05$) (Figure 2). Compared with the model group, Ghrelin group had evidently reduced average optical density of the positive expressions of IL-6 and TNF-α, and the differences were statistically significant ($p<0.05$).

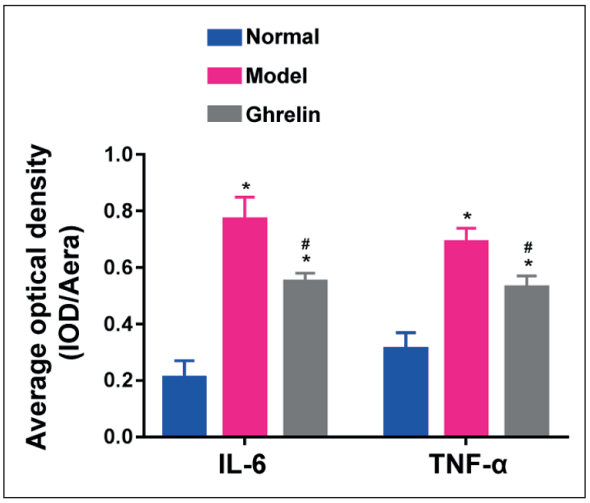

Figure 2. Average optical density of positive expression in each group. Note: *$p<0.05$ *vs.* normal group, #$p<0.05$ *vs.* model group.

WB Assay

The protein expression levels of p-JAK and STAT3 proteins were lower in the normal group but higher in the model group (Figure 3). It was shown in the statistical results (Figure 4) that the model group and Ghrelin group exhibited remarkably increased protein expression levels of p-JAK and STAT3 compared with the normal group, showing statistically significant differences ($p<0.05$). Besides, Ghrelin group manifested notably decreased

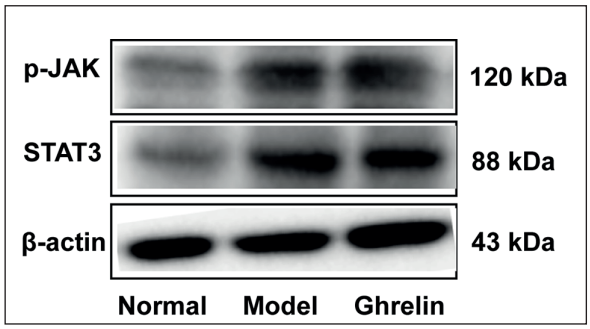

Figure 3. Protein expressions detected via WB.

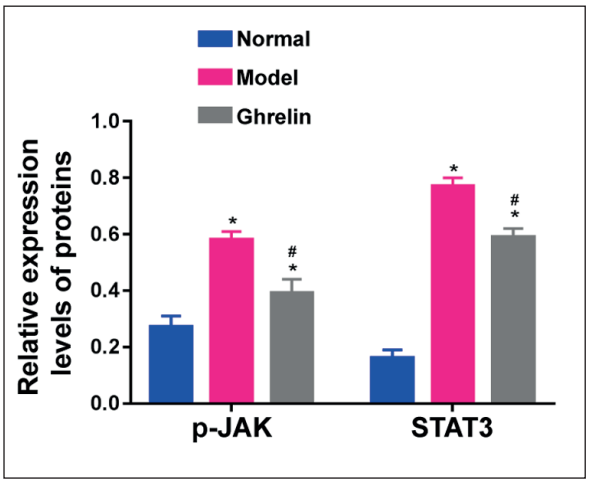

Figure 4. Relative expression levels of proteins in each group. Note: *p<0.05 *vs.* normal group, #p<0.05 *vs.* model group.

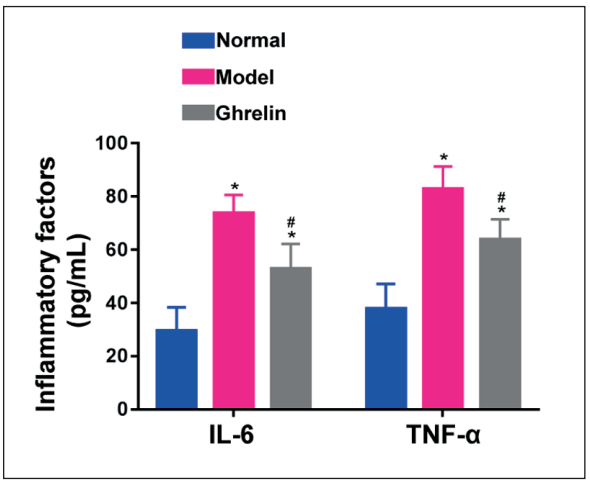

Figure 6. Content of inflammatory factors in each group. Note: *p<0.05 *vs.* normal group, #p<0.05 *vs.* model group.

protein expression levels of p-JAK and STAT3 in comparison with the model group, and there were statistically significant differences (p<0.05).

Expression Levels of Relevant mRNAs Detected Via qPCR

The relative mRNA expression levels of JAK and STAT3 were low in the normal group and fairly high in the model group (Figure 5). The relative mRNA expression levels of JAK and STAT3 were distinctly higher in the model group and Ghrelin group than those in the normal group, with statistically significant differences (p<0.05). Moreover, those levels were evidently lower in Ghrelin group than those in the model group, and the differences were statistically significant (p<0.05).

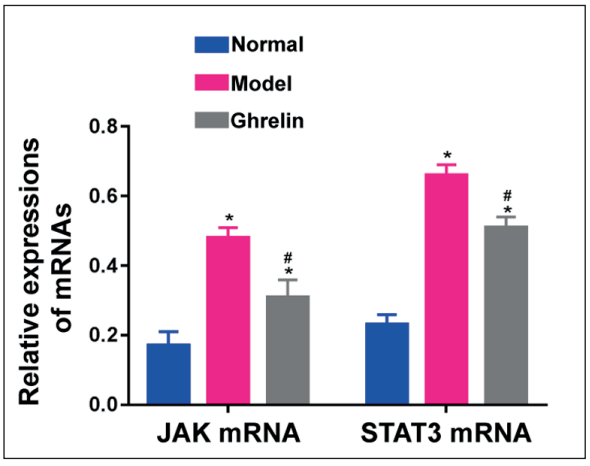

Figure 5. Relative expressions of mRNAs in each group. Note: *p<0.05 *vs.* normal group, #p<0.05 *vs.* model group.

ELISA

As shown in Figure 6, normal group had small content of TNF-α and IL-6, while model group had large amounts of TNF-α and IL-6. The content of TNF-α and IL-6 remarkably raised in the model group and Ghrelin group in comparison with that in the normal group, displaying statistically significant differences (p<0.05). Furthermore, the content of TNF-α and IL-6 declined prominently in the Ghrelin group compared with that in the model group, and the differences were statistically significant (p<0.05).

Cell Apoptosis Detected Via TUNNEL Assay

The positive apoptotic cells were sepia (Figure 7). There were fewer positive apoptotic cells in normal group and more positive apoptotic cells in the remaining groups. According to Figure 8, the apoptosis rate was increased markedly in the remaining groups in comparison with that in the normal group, manifesting statistically significant differences (p<0.05), and it was decreased

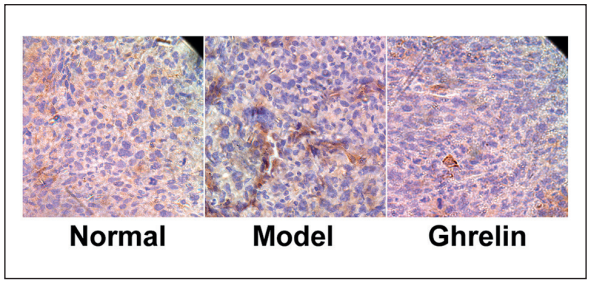

Figure 7. Cell apoptosis detected via TUNNEL (magnification: 200×).

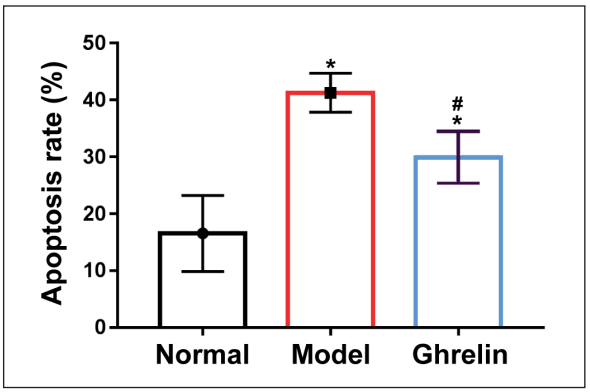

Figure 8. Apoptosis rate in each group. Note: *$p<0.05$ *vs.* normal group, #$p<0.05$ *vs.* model group.

remarkably in the Ghrelin group compared with that in the model group, showing a statistically significant difference ($p<0.05$).

Discussion

Sepsis is a serious systemic infectious disease, and systemic inflammatory response is the most important pathological process and response[9,10]. After the occurrence of sepsis, large quantities of pro-inflammatory cytokines, including IL-6 and TNF-α, are released into the circulation system in the body, which can reach multiple systems and organs to induce secondary inflammatory responses in other systems and organs, further exacerbating the release of local pro-inflammatory cytokines, such as IL-6 and TNF-α in organs to lead to inflammation in several organs and triggering multiple organ dysfunction. As a vital inflammation-associated signaling pathway in organisms, the JAK/STAT signaling pathway plays an important regulatory role in inflammation, that is, it can mediate and aggravate inflammation to form a series of cascades after injury, ultimately resulting in worsening and diffusion of systemic inflammation[11,12]. In-depth studies have illustrated that the JAK/STAT signaling pathway has a crucial function in regulating inflammation and exerts important effects in sepsis onset and disease progression. Besides, it can mediate the transcription and expression of a variety of inflammatory factors in the process of sepsis[13,14]. STAT3, an essential member of the STAT family and a key molecule in the JAK/STAT signaling pathway, can participate in regulating the synthesis and release of many downstream pro-inflammatory cytokines after the JAK/STAT signaling pathway is activated[15,16]. When sepsis occurs in the body, the infection-induced inflammation can cause the synthesis and release of pro-inflammatory cytokines IL-6 and TNF-α, while these cytokines can activate the JAK/STAT signaling pathway to phosphorylate JAK, stimulate STAT-3, and enhance the capability of transcribing IL-6 and TNF-α, thereby aggravating inflammation and finally causing severe systemic inflammation[17-20].

The main novelty of this research is that we first explored the effect and mechanism of Ghrelin on sepsis. We also explained that Ghrelin may protect against sepsis-induced myocardial injury in rats *via* inhibiting the JAK/STAT signaling pathway. These results verified that serious inflammatory response could be observed in cardiac tissues of sepsis model rats, the content of pro-inflammatory substances IL-6 and TNF-α was high in the tissues, and apoptosis occurred in a lot of cells under the action of inflammation. Meanwhile, the JAK/STAT signaling pathway in the cardiac tissues of sepsis model rats was activated, and the proteins and mRNAs of p-JAK and STAT3 were highly expressed, further indicating that the JAK/STAT signaling pathway is involved in the inflammatory response in the cardiac tissues of septic rats, which leads to secondary myocardial injury. Furthermore, Ghrelin, a novel brain-gut polypeptide, had preferable inhibitory effects on the inflammatory response and cell apoptosis in the cardiac tissues of septic rats, reduced the expressions of pro-inflammatory cytokines IL-6 and TNF-α in a better way, and repressed the protein and mRNA expressions of p-JAK and STAT3 at the same time, suggesting that Ghrelin exerts certain inhibitory effects on the JAK/STAT signaling pathway.

Conclusions

The findings of this study show that Ghrelin can inhibit the inflammatory response and apoptosis in the process of myocardial injury in septic rats by repressing the JAK/STAT signaling pathway.

Conflict of Interests

The authors declare that they have no conflict of interest.

References

1) Mantzarlis K, Tsolaki V, Zakynthinos E. Role of oxidative stress and mitochondrial dysfunction in sepsis and potential therapies. Oxid Med Cell Longev 2017; 2017: 5985209.
2) Galley HF. Oxidative stress and mitochondrial dysfunction in sepsis. Br J Anaesth 2011; 107: 57-64.
3) Dellinger RP, Levy MM, Rhodes A, Annane D, Gerlach H, Opal SM, Sevransky JE, Sprung CL, Douglas IS, Jaeschke R, Osborn TM, Nunnally ME, Townsend SR, Reinhart K, Kleinpell RM, Angus DC, Deutschman CS, Machado FR, Rubenfeld GD, Webb S, Beale RJ, Vincent JL, Moreno R. Surviving sepsis campaign: international guidelines for management of severe sepsis and septic shock, 2012. Intensive Care Med 2013; 39: 165-228.
4) Cohen J. The immunopathogenesis of sepsis. Nature 2002; 420: 885-891.
5) Schouten M, Wiersinga WJ, Levi M, van der Poll T. Inflammation, endothelium, and coagulation in sepsis. J Leukoc Biol 2008; 83: 536-545.
6) Hotchkiss RS, Nicholson DW. Apoptosis and caspases regulate death and inflammation in sepsis. Nat Rev Immunol 2006; 6: 813-822.
7) Zundler S, Neurath MF. Integrating immunologic signaling networks: the JAK/STAT pathway in colitis and colitis-associated cancer. Vaccines (Basel) 2016; 4: 5.
8) Kacimi R, Giffard RG, Yenari MA. Endotoxin-activated microglia injure brain derived endothelial cells via NF-kappaB, JAK-STAT and JNK stress kinase pathways. J Inflamm (Lond) 2011; 8: 7.
9) Zhong W, Qian K, Xiong J, Ma K, Wang A, Zou Y. Curcumin alleviates lipopolysaccharide induced sepsis and liver failure by suppression of oxidative stress-related inflammation via PI3K/AKT and NF-kappaB related signaling. Biomed Pharmacother 2016; 83: 302-313.
10) Mira JC, Gentile LF, Mathias BJ, Efron PA, Brakenridge SC, Mohr AM, Moore FA, Moldawer LL. Sepsis pathophysiology, chronic critical illness, and persistent inflammation-immunosuppression and catabolism syndrome. Crit Care Med 2017; 45: 253-262.
11) Zhao H, Liu Z, Shen H, Jin S, Zhang S. Glycyrrhizic acid pretreatment prevents sepsis-induced acute kidney injury via suppressing inflammation, apoptosis and oxidative stress. Eur J Pharmacol 2016; 781: 92-99.
12) Schick MA, Baar W, Flemming S, Schlegel N, Wollborn J, Held C, Schneider R, Brock RW, Roewer N, Wunder C. Sepsis-induced acute kidney injury by standardized colon ascendens stent peritonitis in rats - a simple, reproducible animal model. Intensive Care Med Exp 2014; 2: 34.
13) Roskoski RJ. Janus kinase (JAK) inhibitors in the treatment of inflammatory and neoplastic diseases. Pharmacol Res 2016; 111: 784-803.
14) Banerjee S, Biehl A, Gadina M, Hasni S, Schwartz DM. JAK-STAT signaling as a target for inflammatory and autoimmune diseases: current and future prospects. Drugs 2017; 77: 521-546.
15) Li JM, Zhang H, Zuo YJ. MicroRNA-218 alleviates sepsis inflammation by negatively regulating VOPP1 via JAK/STAT pathway. Eur Rev Med Pharmacol Sci 2018; 22: 5620-5626.
16) Onogawa T, Saito-Taki T, Yamamoto H, Wada T. IL6 trans-signaling promotes functional recovery of hypofunctional phagocytes through STAT3 activation during peritonitis. Inflamm Res 2013; 62: 797-810.
17) Wang A, Xiao Z, Zhou L, Zhang J, Li X, He Q. The protective effect of atractylenolide I on systemic inflammation in the mouse model of sepsis created by cecal ligation and puncture. Pharm Biol 2016; 54: 146-150.
18) Nishi Y, Yoh J, Hiejima H, Kojima M. Structures and molecular forms of the ghrelin-family peptides. Peptides 2011; 32: 2175-2182.
19) Lobo LA, Benjamim CF, Oliveira AC. The interplay between microbiota and inflammation: lessons from peritonitis and sepsis. Clin Transl Immunology 2016; 5: e90.
20) Branco RG, Garcia PC. Ferritin and C-reactive protein as markers of systemic inflammation in sepsis. Pediatr Crit Care Med 2017; 18: 194-196.