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Irisin alleviates renal injury caused by sepsis *via* the NF-κB signaling pathway

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Abstract. – OBJECTIVE: Renal injury caused by sepsis is a difficult point in the field of critical care medicine today, which seriously endangers the health of patients. The aim of our paper was to study the role of irisin in the inflammation and apoptosis of renal injury caused by sepsis and its potential mechanism of action.

MATERIALS AND METHODS: Lipopolysaccharide (LPS) was utilized to establish an acute kidney injury model. HK-2 cells were divided into 3 groups: control group, LPS group, LPS+irisin group. The expression of TNF-α, IL-1β, Bcl-2, and Bax were detected using Western blot. Commercial enzyme-linked immunosorbent assay (ELISA) kits were used to detect the levels of TNF-α, IL-6, and IL-1β in the cell supernatant. The LDH content was detected to observe cell damage. TUNEL staining and flow cytometry were to investigate the apoptosis in three groups. The viability of HK-2 cells was detected using Cell Counting Kit-8 (CCK-8) assay.

RESULTS: After HK-2 cells were treated with LPS, the LDH content in the cell supernatant was greatly increased, and the expression of TNF-α, IL-6, and IL-1β was also significantly increased. However, after treatment with irisin, LDH content and expression of inflammatory factors were significantly suppressed. Similarly, LPS treatment greatly elevated the levels of TNF-α, IL-1β, Bax, p65 and IκKα, as well as inhibited the expression of Bcl-2 and IκB-α. However, irisin treatment reversed these situations. In addition, the number of TUNEL-positive cells and the apoptotic rate were also greatly decreased in LPS+irisin group compared with those in LPS group.

CONCLUSIONS: Irisin could inhibit inflammation and apoptosis of HK-2 cells treated with LPS via the NF-κB pathway.

Key Words:
Irisin, Renal injury, Sepsis, Lipopolysaccharide, NF-κB pathway.

Introduction

Acute kidney injury (AKI) is a clinical syndrome in which a sudden decrease in glomerular filtration rate for a variety of reasons leads to the accumulation of nitrogen-containing products, resulting in a disturbance in the balance of water, electrolyte, acid and base and a rapid development of systemic complications[1]. Sepsis refers to a life-threatening organ dysfunction caused by a host's unbalanced response to an infection. Sepsis is a common critical illness in the intensive care unit with a high mortality rate[2], and affects the kidneys most often; its prevalence was 68.4%[3]. Sepsis AKI has a higher prevalence and more severe kidney damage than non-septic AKI rats. The prevalence of sepsis AKI is 11% to 31%, and it is as high as 41% to 78% in patients with septic shock, and the mortality of patients with sepsis AKI reaches 70%[4]. Therefore, AKI is an independent risk factor for increased risk of sepsis death. Strengthening the prevention and treatment of AKI in sepsis is of great significance for improving the prognosis of these patients.

Body's sepsis can lead to AKI and even multiple organ dysfunction syndrome, which may be related to a series of pathophysiological changes, such as immune disorders, inflammatory response, and apoptosis[5]. Thus, in the treatment of septic induced AKI, inhibition of inflammatory cascade amplification effect of renal tubular epithelial cells and reduction of apoptosis are effective measures to reduce the mortality of septic AKI, and are also the focus and difficulty in the field of critical care medicine[6].

Irisin is a peptide fragment cleaved by the fibronectin type III domain containing protein 5 (FNDC5) and secreted into the blood[7]. Important-

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ly, irisin can promote the conversion of white fat to brown fat and accelerate metabolism, thereby reducing weight, increasing energy release, regulating glucose homeostasis, improving impaired glucose tolerance, and insulin resistance[8]. Irisin can activate AMPK to promote glucose uptake in skeletal muscle, thereby improving insulin sensitivity. It induces uncoupling protein-1 (UCP1) gene expression, and turns white adipocytes into brown adipocytes through p38 protein kinase and extracellular regulatory protein kinase (ERK) 1/2 signal transduction pathway. Irisin activates AMPK-CREBO2 sterol regulatory element-binding transcription factor 2 pathway to inhibit liver cholesterol synthesis[9]; it directly affects adipocytes and inhibits the expression of inflammatory cytokines such as TNF-α and IL-6[10]. Moreover, irisin regulates macrophage activity by reducing excess oxygen, which may indicate its potential anti-inflammatory properties[11]. Thus, irisin functions in obesity, diabetes mellitus, heart disease, osteogenesis, bone metabolism, and chronic kidney disease. However, few articles have reported the role of irisin in AKI, especially AKI induced by sepsis.

In our study, LPS-induced HK-2 cell injury model was adopted to investigate whether irisin can play an anti-apoptotic and anti-inflammatory role and its potential mechanism of action. Our results showed that irisin may provide a potential new treatment for AKI caused by sepsis.

Materials and Methods

Cell Culture

HK-2 cells (Procell, Wuhan, China) were cultured in Dulbecco's Modified Eagle's Medium/F-12 (DMEM/F-12; Life Technology, Wuhan, China) containing 10% fetal bovine serum (FBS; Life Technology, Wuhan, China) and 1% penicillin/streptomycin (Life Technology, Wuhan, China), in 37°C, 5% CO_2 incubator. The cell medium was changed once a day and cell passage was performed when the cell confluence reached about 80%. When the HK-2 cells grew to the appropriate density, they were treated with LPS (Sigma-Aldrich, St. Louis, MO, USA) and irisin (Phoenix Pharmaceuticals, Princeton, NJ, USA).

LDH Levels

The content of LDH in HK-2 cell supernatant was measured by Cytotoxicity LDH Assay Kit (Dojindo Molecular Technologies, Kumamoto, Japan).

Cell Counting Kit-8 (CCK-8) Assay

We first took log phase HK-2 cells, adjusted the cell suspension concentration to about 5×10^4 /mL, and added 100 μL into each well of 96-well plate. Then, the supernatant in the well was discarded, and LPS or irisin was added at different concentrations. The cell plates were placed in the incubator for 24 hours. Then, the supernatant was discarded and the CCK-8 reagent (10 μL; MCE, Monmouth Junction, NJ, USA) and serum-free medium (90 μL; Life Technology, Wuhan, China) were added for 30 min. The optical density (OD) value of each well was measured by a microplate reader.

Western Blot

The number of cells in each well of 6-well plates was 1×10^5. The HK-2 cells were treated with LPS and/or irisin. The cells were cultured under the same conditions for 24 hours and then collected. The total proteins in each group were extracted with a protein extraction kit (Camilo Biological, Nanjing, China). The total protein was quantified by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA), and the protein was denatured by boiling for 10 min. 15 μL of the sample was electrophoresed on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; Beyotime Biotechnology, Shanghai, China) and then transferred to polyvinylidene difluoride (PVDF; EpiZyme, Shanghai, China), followed by 5% bovine serum albumin (BSA; Beyotime Biotechnology, Shanghai, China) to block the band. The band was then incubated with primary antibody [Bax, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; Bcl-2, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; TNF-α, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; IL-1β, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; IκB-α, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; p65, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; IκKα, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Abcam, Cambridge, MA, USA, Rabbit, 1:1000] at 4°C overnight, and then incubated with secondary antibody. Finally, the gel imaging system enhanced chemiluminescence (ECL) was used for development.

TNF-α and IL-6 and IL-1β Contents

The supernatants of the three groups of cells were taken and the contents of TNF-α and IL-

6 and IL-1β were detected by TNF-α and IL-6 and IL-1β enzyme-linked immunosorbent assay (ELISA) kits (Dojindo Molecular Technologies, Kumamoto, Japan), respectively.

TUNEL Staining

HK-2 cells were plated in 24-well plates and subjected to TUNEL staining using TUNEL kit (Roche, Basel, Switzerland) after treatment with LPS and/or irisin for 24 hours according to the previously mentioned procedure.

Flow Cytometry

HK-2 cells and supernatant were collected, then, centrifuged at 3000 r/min for 5 minutes, washed with PBS and centrifuged twice, and resuspended in 500 μL Binding Buffer. Annexin V-FITC (fluorescein isothiocyanate; KeyGen, Shanghai, China) and Propidium Iodide (PI; KeyGen, Shanghai, China) were added with 5 μL each, and flow cytometry quantitative detection (generally no more than 1 h) was performed with FACScan immediately after reaction in the dark for 5 minutes.

Statistical Analysis

Data were expressed as $\bar{x} \pm s$ and were plotted using GraphPad Prism5 software (La Jolla, CA, USA). The differences between the two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). $p<0.05$ indicated the significant difference.

Results

Irisin Protected LPS-Induced Cell Injury

We first plated HK-2 cells into 96-well plates, and added different concentrations of LPS (0, 100, 300, 500, 700, 1000 ng/mL), and used CCK-8 assay to measure cell viability after 24 hours. When the concentration of LPS was 500 ng/mL, the cell viability was about 0.5, so this concentration was selected for subsequent experiments (Figure 1A). Then, 500 ng/mL of LPS was added to each well (except for the control group), and different concentrations of irisin (0, 1, 2, 3, 4, 5 μg/mL) were simultaneously added, and cell viability was measured after 24 hours. When the irisin concentration was 4 μg/mL and 5 μg/mL, the cell viability reached a maximum, so the concentration of 4 μg/mL was selected for further study (Figure 1B). We also tested the LDH content in the cell supernatant of each group and found that the LDH content in the LPS group increased remarkably, while the LDH content in the LPS + irisin group markedly decreased (Figure 1C).

Irisin Inhibited LPS-Induced Inflammation

After the above treatment of HK-2 cells, we extracted total protein and performed Western blot to detect TNF-α and IL-1β expression (Figure 2A). After treatment of LPS, the levels of TNF-α and IL-1β were greatly upregulated, but the treatment of irisin downregulated the expression of these two proteins (Figure 2B). In addition, we tested the expression of inflammatory factors (TNF-α, IL-1β, IL-6) in the cell supernatant of each group using commercial kits. We found that irisin can significantly reverse the increase of in-

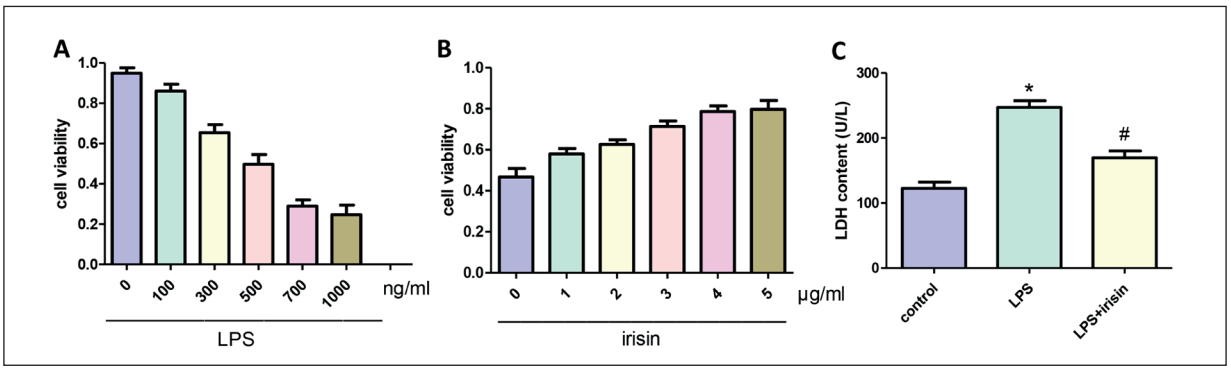

Figure 1. Irisin protected LPS-induced cell injury. **A,** CCK-8 assay showed HK-2 cells viability at different concentrations of LPS. **B,** CCK-8 assay showed HK-2 cell viability after addition of different concentrations of Irisin in LPS-treated HK-2 cells. **C,** The LDH content in the cell supernatants of each group was detected using an LDH detection kit ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS, n=3).

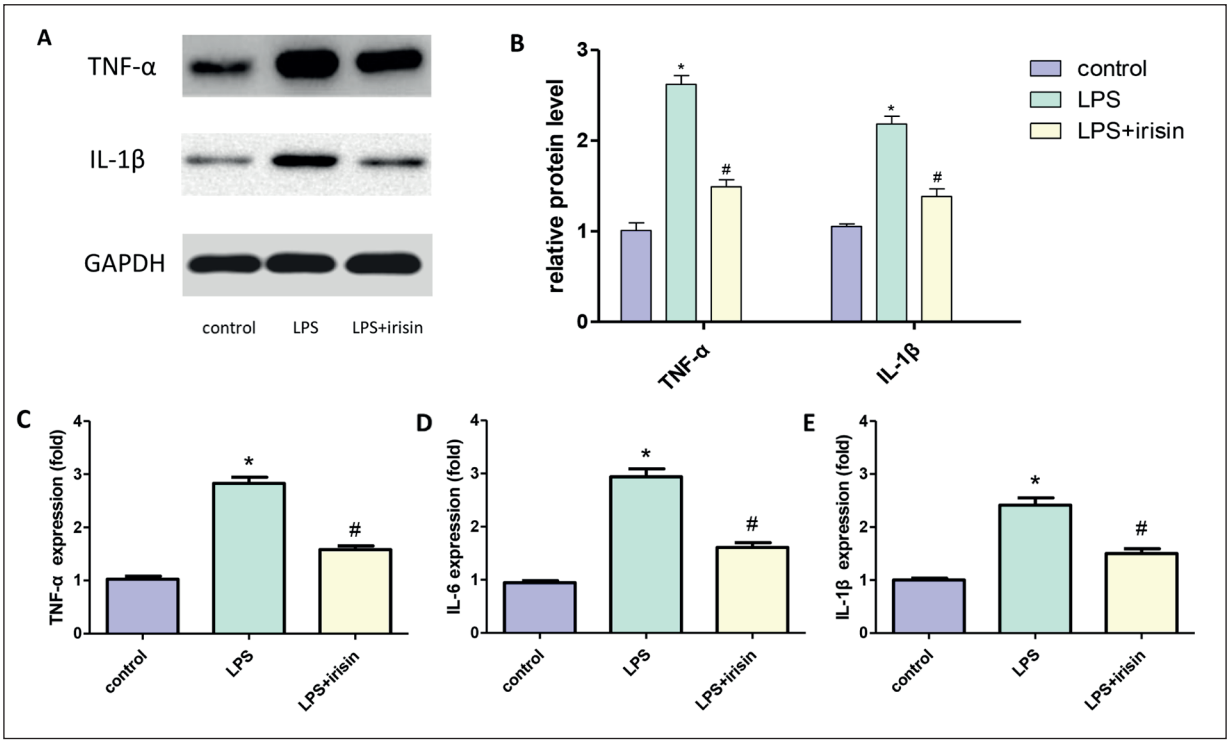

Figure 2. Irisin inhibited LPS-induced inflammation in HK-2 cells. **A,** Protein expression results of TNF-α and IL-1β in three groups were determined by Western blotting. GAPDH was used as an internal control. **B,** Statistical results of expression of TNF-α and IL-1β ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS, n=3). **C-E,** ELISA assay was used to detect the protein expression of TNF-α, IL-6, and IL-1β in the three groups ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS, n=3).

flammatory factors in each group caused by LPS (Figure 2C-2E). These results indicated that irisin can inhibit the inflammatory response caused by LPS in HK-2 cells.

Irisin Inhibited LPS-Induced Apoptosis

To assess the effect of irisin on apoptosis, we first examined the levels of Bax and Bcl-2 (Figure 3A). As it can be seen from Figure 3B, LPS can greatly reduce the Bcl-2 expression and increase the Bax expression, but irisin could reverse the expression of both proteins. The results of TUNEL staining suggested that irisin can greatly reduce the number of TUNEL positive cells (Figure 3C and 3D). The results of flow cytometry also suggested that irisin can reverse the increase of apoptosis rate of HK-2 cells caused by LPS (Figure 3E and 3F). These results showed that irisin can inhibit apoptosis caused by LPS in HK-2 cells.

Irisin Inhibited the NF-κB Pathway

The NF-κB pathway plays a very important role in regulating apoptosis and inflammation, so we examined the NF-κB pathway in different treatment groups by Western blot (Figure 4A). We can see from Figure 4B that the expression of p65 and IκB kinase α (IκKα) in LPS treatment group were significantly increased, while the expression of inflammatory inhibitor NF-κBα (IκB-α) was greatly decreased, compared with the control group. After treatment with irisin, the expression of p65 and IκKα were decreased, while the expression level of IκB-α increased compared to the LPS group. These results suggested that irisin can inhibit the NF-κB pathway.

Discussion

The pathogenesis of sepsis-related kidney injury is complex and multifactorial, and the specific mechanism is still not fully understood. In addition to the special hemodynamic factors of the kidney, endothelial dysfunction, renal parenchymal inflammatory cell infiltration, glomerular thrombosis, and congestive changes of necrotic cells are also important mechanisms that cause sepsis-related AKI[12,13].

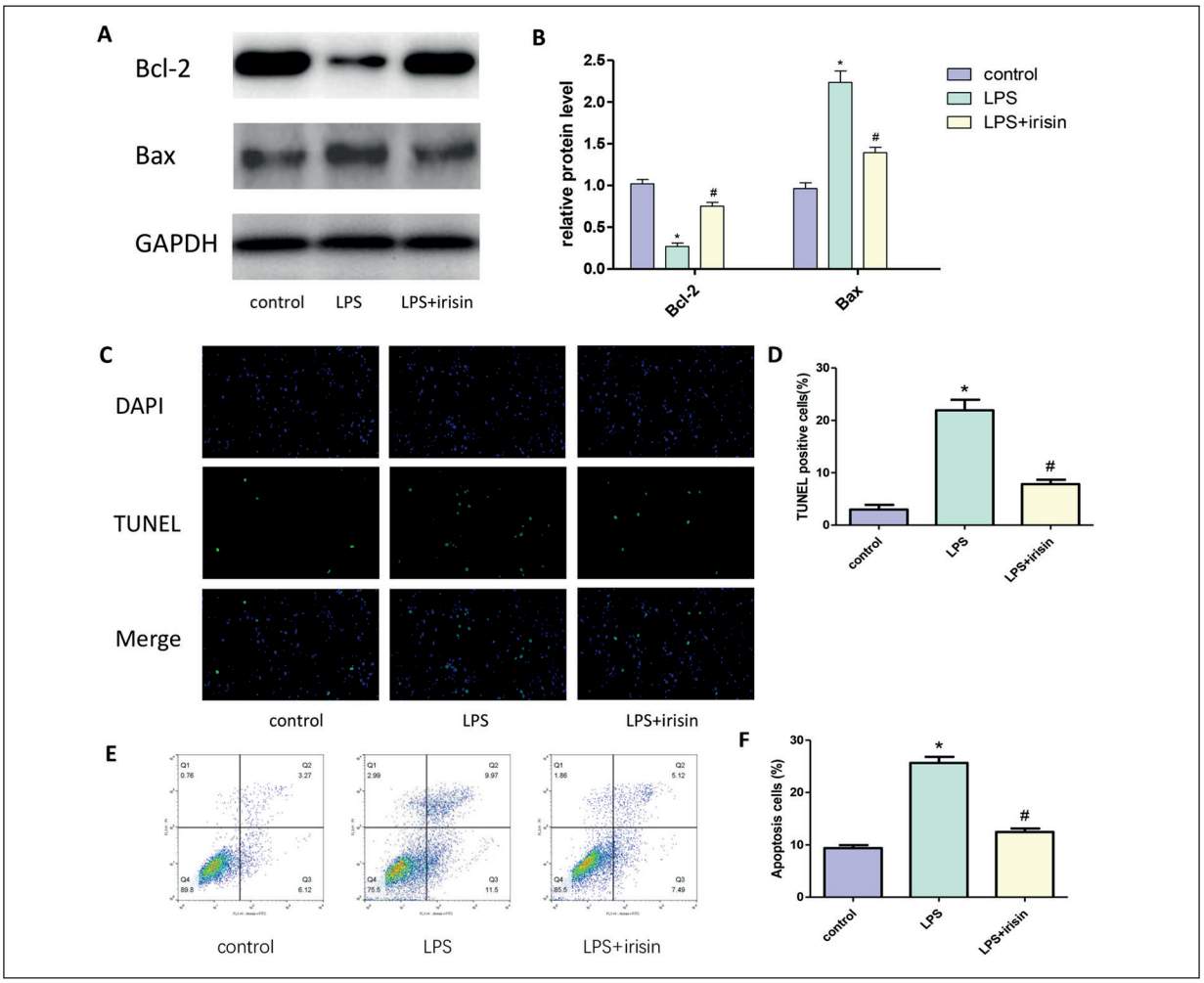

Figure 3. Irisin inhibited LPS-induced apoptosis in HK-2 cells. **A,** Protein expression results of Bcl-2 and Bax in three groups were determined by Western blotting. GAPDH was used as an internal control. **B,** Statistical results of expression of Bcl-2 and Bax ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS, n=3). **C,** TUNEL staining showed that irisin can significantly reduce the increase of HK-2 cell apoptosis caused by LPS (magnification: 400×). **D,** Statistical analysis of TUNEL staining ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS, n=3). **E,** The flow cytometry showed that the apoptotic rate of LPS treatment group increased, and decreased in LPS+irisin group. **F,** Statistical results of apoptotic rate of HK-2 cells ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS, n=3).

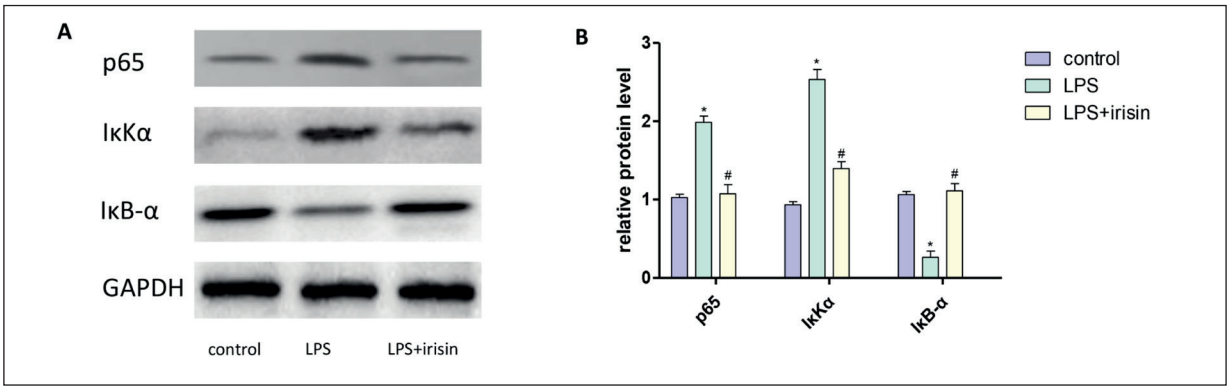

Figure 4. Irisin inhibited the NF-κB pathway. **A,** The expression of IκB-α, p65 and IκKα was determined by Western blot analysis. GAPDH was used as an internal control. **B,** Statistical results of expression of IκB-α, p65 and IκKα ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS, n=3).

Renal hemodynamic changes during sepsis, cytokine-mediated nitric oxide (NO) release, which reduces systemic vascular resistance, reduces cardiac preload, and activates the neurohumoral axis, renin-angiotensin-aldosterone system, and sympathetic nervous system. These results lead to the release of vasopressin, renal vasoconstriction, decreased renal blood flow, and decreased renal perfusion, leading to AKI[14]. Sepsis is a systemic inflammatory response caused by infection. Pathogens invade the body and cause tissue damage, releasing danger signals, including the pathogen-associated molecular pattern (PAMP) of the infection itself and the damage-associated molecular pattern (DAMP) of the injured tissue. Immune cells, endothelial cells, and renal tubular epithelial cells recognize PAMP and DAMP through pattern receptors, initiate a natural immune inflammatory response, and release a lot of inflammatory mediators, such as IL-1, IL-6, and tumor necrosis factor[15]. The massive release of these inflammatory factors forms a cytokine storm, which leads to continuous activation of the immune system, upregulation of adhesion molecules, release of more pro-inflammatory mediators and reactive oxygen species (ROS), microvascular dysfunction, causing hypoxia and inflammatory damage to tissues[16,17]. As one of the target organs attacked by inflammatory cytokines, the kidney is extremely vulnerable to damage.

Normal apoptosis is important for maintaining cell integrity and homeostasis. In sepsis-associated AKI, caspase 3 activation was increased, apoptosis-inducing factors and cytochrome C were released, Bcl-2 gene-related X protein was activated, and Bcl-2 gene was deleted. Furthermore, apoptotic bodies and *in situ* terminal transferase labeling staining were positive in renal tubular cells, indicating that apoptosis exists and plays an important role in the process of AKI caused by sepsis, while renal cell apoptosis is rare in non-septic AKI[18,19].

The anti-inflammatory and anti-apoptotic effects of irisin have been understood, but its role in sepsis AKI has been rarely studied. Since inflammation and apoptosis play a pivotal role in the occurrence and development of sepsis renal injury, we focused on the role of irisin in the inflammation and apoptosis of sepsis AKI. The results of our research revealed that irisin exerts a powerful anti-inflammatory and anti-apoptotic effect in an *in vitro* model of acute kidney injury in sepsis, which surprises us. However, more investigations are still needed to further prove the role of irisin in *in vivo* models.

Conclusions

In this study, we constructed an *in vitro* model of sepsis induced AKI using LPS, and found that the expression of TNF-α, IL-1β, Bax increased, and the expression of Bcl-2 decreased, demonstrating that LPS promotes inflammation and apoptosis of HK-2 cells. When treated with irisin, the inflammation and apoptosis of the cells were significantly reversed. Moreover, irisin greatly increased the expression of p65 and IκKα and decreased IκB-α expression. Therefore, we suggest that irisin inhibits inflammation and apoptosis of HK-2 cells *via* inhibiting the NF-κB pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Skube SJ, Katz SA, Chipman JG, Tignanelli CJ. Acute kidney injury and sepsis. Surg Infect (Larchmt) 2018; 19: 216-224.
2) Seymour CW, Liu VX, Iwashyna TJ, Brunkhorst FM, Rea TD, Scherag A, Rubenfeld G, Kahn JM, Shankar-Hari M, Singer M, Deutschman CS, Escobar GJ, Angus DC. Assessment of clinical criteria for sepsis: for the third international consensus definitions for sepsis and septic shock (sepsis-3). JAMA 2016; 315: 762-774.
3) Zeng X, McMahon GM, Brunelli SM, Bates DW, Waikar SS. Incidence, outcomes, and comparisons across definitions of AKI in hospitalized individuals. Clin J Am Soc Nephrol 2014; 9: 12-20.
4) Peng Q, Zhang L, Ai Y, Zhang L. Epidemiology of acute kidney injury in intensive care septic patients based on the KDIGO guidelines. Chin Med J (Engl) 2014; 127: 1820-1826.
5) Zarbock A, Gomez H, Kellum JA. Sepsis-induced acute kidney injury revisited: pathophysiology, prevention and future therapies. Curr Opin Crit Care 2014; 20: 588-595.
6) Prowle JR, Bellomo R. Sepsis-associated acute kidney injury: macrohemodynamic and microhemodynamic alterations in the renal circulation. Semin Nephrol 2015; 35: 64-74.
7) Bostrom P, Wu J, Jedrychowski MP, Korde A, Ye L, Lo JC, Rasbach KA, Bostrom EA, Choi JH, Long JZ, Ka-

JIMURA S, ZINGARETTI MC, VIND BF, TU H, CINTI S, HOJLUND K, GYGI SP, SPIEGELMAN BM. A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. Nature 2012; 481: 463-468.

8) PERAKAKIS N, TRIANTAFYLLOU GA, FERNANDEZ-REAL JM, HUH JY, PARK KH, SEUFERT J, MANTZOROS CS. Physiology and role of irisin in glucose homeostasis. Nat Rev Endocrinol 2017; 13: 324-337.

9) TANG H, YU R, LIU S, HUWATIBIEKE B, LI Z, ZHANG W. Irisin inhibits hepatic cholesterol synthesis via AMPK-SREBP2 signaling. EBioMedicine 2016; 6: 139-148.

10) MAZUR-BIALY AI, BILSKI J, POCHEC E, BRZOZOWSKI T. New insight into the direct anti-inflammatory activity of a myokine irisin against proinflammatory activation of adipocytes. Implication for exercise in obesity. J Physiol Pharmacol 2017; 68: 243-251.

11) MAZUR-BIALY AI. Irisin acts as a regulator of macrophages host defense. Life Sci 2017; 176: 21-25.

12) SHUM HP, YAN WW, CHAN TM. Recent knowledge on the pathophysiology of septic acute kidney injury: A narrative review. J Crit Care 2016; 31: 82-89.

13) BILGILI B, HALILOGLU M, CINEL I. Sepsis and acute kidney injury. Turk J Anaesthesiol Reanim 2014; 42: 294-301.

14) MAYEUX PR, MACMILLAN-CROW LA. Pharmacological targets in the renal peritubular microenvironment: implications for therapy for sepsis-induced acute kidney injury. Pharmacol Ther 2012; 134: 139-155.

15) PRINCE LR, WHYTE MK, SABROE I, PARKER LC. The role of TLRs in neutrophil activation. Curr Opin Pharmacol 2011; 11: 397-403.

16) ZHANG Y, SU X, ZOU F, XU T, PAN P, HU C. Toll-like receptor-4 deficiency alleviates chronic intermittent hypoxia-induced renal injury, inflammation, and fibrosis. Sleep Breath 2019; 23: 503-513.

17) BAGSHAW SM, LAPINSKY S, DIAL S, ARABI Y, DODEK P, WOOD G, ELLIS P, GUZMAN J, MARSHALL J, PARRILLO JE, SKROBIK Y, KUMAR A. Acute kidney injury in septic shock: clinical outcomes and impact of duration of hypotension prior to initiation of antimicrobial therapy. Intensive Care Med 2009; 35: 871-881.

18) LEROLLE N, NOCHY D, GUEROT E, BRUNEVAL P, FAGON JY, DIEHL JL, HILL G. Histopathology of septic shock induced acute kidney injury: apoptosis and leukocytic infiltration. Intensive Care Med 2010; 36: 471-478.

19) JACOBS R, HONORE PM, JOANNES-BOYAU O, BOER W, DE REGT J, DE WAELE E, COLLIN V, SPAPEN HD. Septic acute kidney injury: the culprit is inflammatory apoptosis rather than ischemic necrosis. Blood Purif 2011; 32: 262-265.