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Original Article

Expression of FoxO3a in sepsis mice and its association with lymphocyte apoptosis

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Abstract

This study investigated forkhead box O3a (FoxO3a) expression in peripheral blood of sepsis mice and its correlation with lymphocyte apoptosis. Sixty male C57 mice were randomly assigned to sham, model, and intervention groups. Sepsis was induced via cecal ligation in the model and intervention groups, while sham mice underwent similar procedures excluding cecal ligation. Apoptosis proteins in lymphocytes were assessed by Western blotting, reactive oxygen species (ROS) levels by 2,7-Dichlorodi-hydrofluorescein diacetate (DCFH-DA), and serum interleukin-1β (IL-1β) and IL-10 content. The model group exhibited elevated mortality, increased lymphocyte apoptosis, higher Caspase3 expression, and lower Bcl-2/Bax ratio compared to sham and intervention groups. Additionally, the model group displayed decreased serum IL-10, elevated IL-1β, heightened lymphocytic ROS, reduced FoxO3a expression, and increased levels of p-FoxO3a, p-PI3K, and p-Akt compared to sham. In sepsis mice, inhibited FoxO3a signaling in lymphocytes leads to enhanced apoptosis, elevated ROS, and immune cell dysfunction, contributing to increased mortality.

Keywords: Sepsis, Lymphocytes, FoxO3a, Apoptosis

1. Introduction

Sepsis, marked by its acute onset, stands as a formidable threat, representing a major cause of mortality among infection patients with the potential to progress to the devastating condition of septic shock [1]. Extensive research substantiates that apoptosis, a highly regulated form of programmed cell death, plays a pivotal role in the pathophysiology of sepsis, contributing significantly to the development of septic shock, multiple organ failure, and eventual demise [2, 3].

The intricate relationship between apoptosis and immune cell dysfunction is particularly noteworthy. Augmented apoptosis of immune cells, both in the adaptive and innate immune systems, emerges as a key mediator of immunosuppression, thereby intensifying the severity of sepsis [4]. This phenomenon is particularly pronounced in vital immune organs such as the spleen and within the systemic circulation, where apoptotic immune cells become engulfed by macrophages, further exacerbating the immunosuppressive state and disrupting the delicate balance of immune responses in sepsis [4].

Among various immune cell types, lymphocyte apoptosis is a predominant and consequential event. Abnormalities in lymphocyte apoptosis have been identified as a leading factor in the development of immunosuppression, resulting in organ damage and a markedly increased mortality rate among sepsis patients [5]. It is within this context that the role of Forkhead box O3a (FoxO3a) comes to the forefront.

FoxO3a, a significant member of the forkhead protein family, serves as a critical transcription factor intricately involved in the processes of cell autophagy and apoptosis. Its ability to interact with NF-κB in the cytoplasm adds another layer of complexity, as this interaction inhibits NF-κB's nuclear translocation, thereby modulating inflammatory signal transduction and reducing the inflammatory response in vivo [6, 7]. Beyond its role in inflammation, FoxO3a actively participates in the metabolic, survival, and antioxidant processes across various cell types. This multifaceted engagement contributes to cellular homeostasis, actively protecting cells and tissues from deleterious effects [8].

Despite the importance of FoxO3a in cellular processes and the limited understanding of its involvement in sepsis, there remains a noticeable gap in research regarding the association between FoxO3a expression and lymphocyte apoptosis in sepsis. Thus, this study endeavors to bridge this gap by delving into the intricate interplay between

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apoptosis and FoxO3a expression in sepsis mice. Through the establishment of a robust mouse model of sepsis, we aim to unearth critical insights that may serve as a theoretical foundation for unraveling the intricate pathogenesis of sepsis and pave the way for potential therapeutic interventions.

2. Materials and methods

2.1. Instruments and materials

IturinA (Sigma, St. Louis, MO, USA), pentobarbital sodium (Sigma, St. Louis, MO, USA), Dulbecco's modified Eagle medium (DMEM) (Hyclone, South Logan, UT, USA), reactive oxygen species (ROS) assay kit (Beyotime, Shanghai, China), interleukin-1β (IL-1β) and IL-10 enzyme-linked immunosorbent assay (ELISA) kits (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China), apoptosis assay kit (R&D, Minneapolis, MN, USA), bicinchoninic acid (BCA) protein quantitation kit (Invitrogen, Carlsbad, CA, USA), polyvinylidene fluoride (PVDF) membrane (Merck, Danvers, MA, USA), rabbit anti-B-cell lymphoma-2 (Bcl-2), rabbit anti-Bcl-2 associated X protein (Bax), rabbit anti-FoxO3a, rabbit anti-p-FoxO3a, rabbit anti-p-phosphatidylinositol 3-hydroxy kinase (PI3K), rabbit anti-p-protein kinase B (Akt), rabbit anti-Caspase3 and rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Danvers, MA, USA), ECL solution (Invitrogen, Carlsbad, CA, USA), and other related instruments and reagents described in the relevant sections.

2.2. Laboratory animals and grouping

Healthy male C57 mice weighing 20-25 g and aged 8-12 weeks old were adaptively fed for 7 d under the temperature of (25±2)°C, humidity of (45±3)% and regular circadian rhythm, and had free access to food and water. The mice were randomly divided into sham group (cecal ligation was not performed and the remaining operations were the same as those in model group), model group (sepsis model was established via cecal ligation) and intervention group [the FoxO3a agonist IturinA (5 mg/kg) was intraperitoneally injected at half an hour before cecal ligation]. At 24 h after surgery, the peripheral blood was drawn from 5 mice in each group, and the plasma and lymphocytes were separated and stored for later use. After 7 d, the survival status of the remaining mice was observed, and the survival rate was calculated. This study was approved by the Animal Ethics Committee of Shanxi Medical University Animal Center.

2.3. Establishment of mouse model of sepsis

The mice were deprived of food for 24 h before surgery. After anesthesia via intraperitoneal injection of pentobarbital sodium solution (1 mg/mL), the abdomen was disinfected with medical aseptic cotton, depilated and disinfected again. The skin was cut open along the midline of the abdomen to expose the abdominal cavity. Then the content of cecum at the left lower abdomen was squeezed to the distal end, and the cecum was ligated using #4 suture at 1/3 of the end (avoid ligating the ileum and cecum mesenteric vessels). The 21-gauge needle was used to perforate equidistantly, and a small amount of content was squeezed out. Then the cecum was placed back into the abdominal cavity, and the abdominal wall incision and skin were sutured layer by layer. The surgical area was disinfected again with medical alcohol [9].

2.4. Separation of lymphocytes

At 24 h after surgery, the eyeball was removed, and the peripheral blood was drawn and stored in the heparin tube for later use, followed by dilution with appropriate amount of phosphate-buffered saline (PBS) (1:1) in a super clean bench. 0.6 mL of lymphocyte separation medium was added into the centrifuge tube, and 0.6 mL of diluted blood was slowly added into the upper layer of separation medium, followed by centrifugation at 1200 g and 25°C for 30 min. Then the "white membrane" between separation medium and plasma was transferred into a new centrifuge tube using a pipette, mixed evenly with an appropriate amount of PBS, and centrifuged at 2500 g for 10 min. After the supernatant was discarded, the lymphocytes were obtained, and the remaining plasma was stored for later use.

2.5. Detection of lymphocyte apoptosis level via flow cytometry

The lymphocyte apoptosis level was detected via flow cytometry, as follows: An appropriate amount of buffer, PI and Annexin V were added to the pre-treated lymphocytes for Annexin V-FITC/PI apoptosis staining in the dark. After standing in the dark for 10 min, the cells were resuspended with 2 mL of pre-cooled PBS, blown over and mixed evenly, followed by flow cytometry, in which the Annexin V-FITC luminescence was detected in FL1 channel and the PI luminescence was detected in FL3 channel.

2.6. Detection of ROS content in lymphocytes via 2,7-dichlorodi-hydrofluorescein diacetate (DCFH-DA)

The pretreated lymphocytes were diluted with PBS to 1×10^6-1×10^7/mL, added with an appropriate amount of serum-free medium containing DCFH-DA, and incubated in an incubator with 5% CO_2 at 37°C for 20 min, during which the mixture was mixed evenly every 5 min so that the cells were sufficiently contacted with the probe. Then the cells were washed with serum-free DMEM 3 times to remove unabsorbed DCFH-DA, and the ROS content in lymphocytes was measured via flow cytometry.

2.7. Determination of content of serum inflammatory factors via ELISA

The content of serum inflammatory factors IL-1β and IL-10 in each group was determined using ELISA kits. The serum was isolated in each group strictly according to the instructions of kits, and the optical density (OD) was measured at 450 nm using a microplate reader. Then the standard curves were plotted, based on which the content of serum inflammatory factors IL-1β and IL-10 was calculated.

2.8. Detection of related protein expression levels using Western blotting

The protein in lymphocytes was quantified using the BCA protein quantification kit. After the loading buffer at an equal concentration was prepared, the protein was separated via SDS-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane. Then the band was cut according to the molecular weight of target proteins, sealed with freshly-prepared skim milk powder at room temperature for 2 h, washed with tris buffered sa-

line-tween (TBST) for 3 times, and incubated with the monoclonal antibodies of Caspase3, Bcl-2, Bax, FxoO3a, p-FxoO3a, p-PI3K and p-Akt at 4°C overnight. After washing with TBST 3 times, the band was incubated again with the secondary antibodies at room temperature for 1 h, and washed again with TBST 3 times. Then an appropriate amount of freshly prepared ECL solution (solution A and solution B were mixed evenly at 1:1) was dropwise added onto the protein band in the darkroom for image development and fixation. After scanning, the gray value was analyzed using the ImageJ software.

2.9. Effect of sepsis on survival rate of mice

At 7 d after surgery, survival status of mice in each group was recorded, and the survival rate was calculated.

2.10. Statistical analysis

The data in this study were expressed as mean ± standard deviation. Statistical Product and Service Solutions (SPSS) 22.0 software (SPSS Inc., Chicago, IL, USA) was used for the data processing. Analysis of variance was performed for the comparison among groups. Bonferroni's method was adopted for pairwise comparison in the case of homogeneity of variance, while Welch's method was adopted in the case of heterogeneity of variance. Dunnett's T3 test was adopted for multiple comparisons.

3. Results

3.1. Comparison of 7-day survival rate of mice

The 7-day survival rate of mice in each group was recorded. As shown in Figure 1, the 7-day survival rate was significantly lower in model group than in sham group (P<0.01), while it was substantially higher in intervention group than in model group (P<0.01).

3.2. Lymphocyte apoptosis level

The lymphocyte apoptosis level in each group was detected via flow cytometry. As shown in Figure 2, the lymphocyte apoptosis level was substantially higher in model group than in sham group (P<0.01), while it was lower in intervention group than in model group (P<0.01).

3.3. Expression levels of apoptosis-related proteins

The expression levels of apoptosis-related proteins in lymphocytes in each group were determined using Western blotting. The results showed that compared with sham group, model group had evidently increased expression of Caspase3 and decreased Bcl-2/Bax in lymphocytes. After treatment with IturinA, the expression level of Caspase3 declined and Bcl-2/Bax evidently rose (Figure 3).

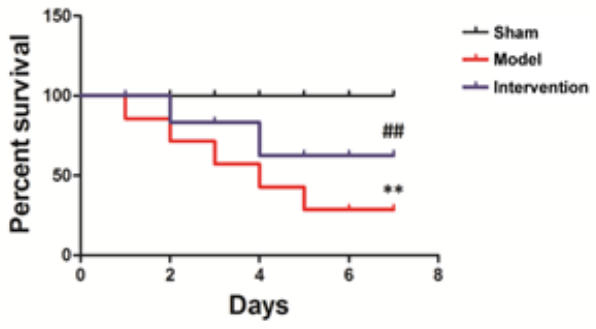

Fig. 1. Comparison of 7-day survival rate of mice. The 7-day survival rate is significantly lower in model group than that in sham group, while it is notably higher in intervention group than that in model group. **P<0.01 *vs.* sham group, ##P<0.01 *vs.* model group.

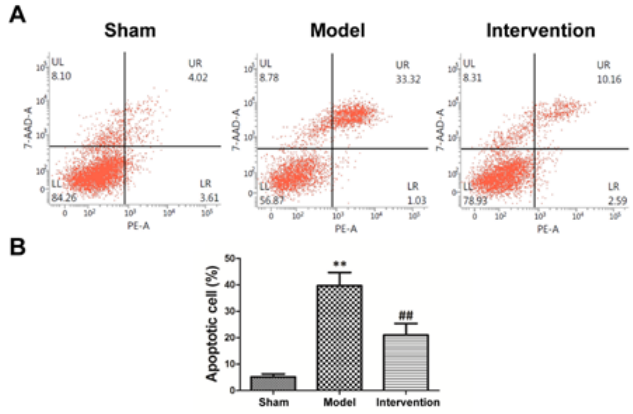

Fig. 2. Lymphocyte apoptosis level in each group. The lymphocyte apoptosis level is higher in model group than in sham group, while it is lower in intervention group than in model group. **P<0.01 *vs.* sham group, ##P<0.01 *vs.* model group.

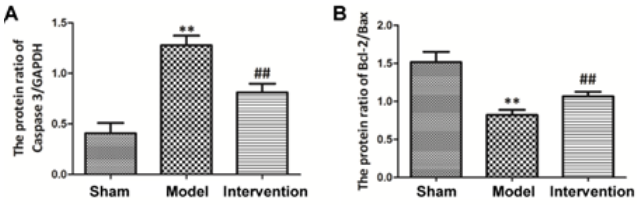

Fig. 3. Expression levels of apoptosis-related proteins. In model group, the expression level of Caspase3 is significantly higher than that in sham group and intervention group, while that of Bcl-2/Bax is significantly lower than that in sham group and intervention group. **P<0.01 *vs.* sham group, ##P<0.01 *vs.* model group.

3.4. Content of ROS in lymphocytes

The content of ROS in lymphocytes was determined using DCFH-DA. It was found that the content of ROS in lymphocytes was remarkably increased in model group compared with that in sham group (P<0.01), while it was notably decreased after treatment with IturinA (P<0.01) (Figure 4).

3.5. Levels of serum inflammatory factors

The levels of serum inflammatory factors in each group were detected using ELISA kits. The results revealed that the level of serum IL-1β was higher and that of IL-10 was lower in model group than in sham group (P<0.01, P<0.01). After treatment with IturinA, the level of serum IL-1β declined and that of IL-10 obviously rose in sepsis mice (P<0.01, P<0.01) (Figure 5).

3.6. Expression levels of FoxO3a signaling pathway-related proteins detected via Western blotting

The expression levels of FoxO3a signaling pathway-related proteins in sepsis mice were detected via Western blotting. It was observed that compared with sham group, model group had significantly lower expression of FoxO3a (P<0.01), and significantly higher expressions of p-FoxO3a, p-PI3K and p-Akt (P<0.01). After treatment with IturinA, the expression of FoxO3a significantly rose

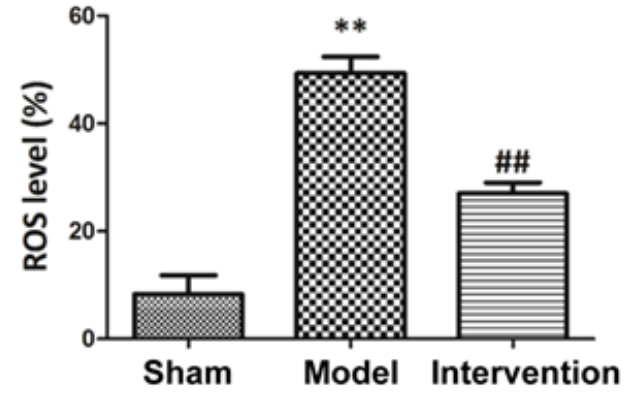

Fig. 4. Content of ROS in lymphocytes determined using DCFH-DA. The content of ROS in lymphocytes is remarkably higher in model group than in sham group and intervention group. **P<0.01 *vs.* sham group, ##P<0.01 *vs.* model group.

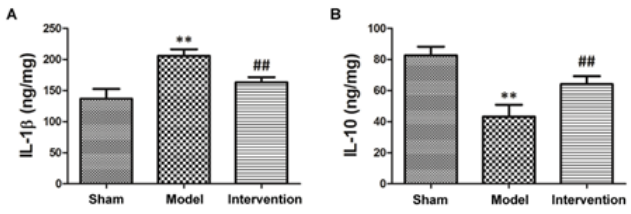

Fig. 5. Levels of serum inflammatory factors detected using ELISA. In model group, the level of serum IL-1β is higher and that of IL-10 is lower than those in sham group and intervention group. **P<0.01 *vs.* sham group, ##P<0.01 *vs.* model group.

(P<0.01), and the expressions of p-FoxO3a, p-PI3K and p-Akt considerably declined (P<0.01) (Figure 6).

4. Discussion

Apoptosis is the active, programmed and energy-consuming cell death process, and exogenous and endogenous stimuli can initiate intracellular apoptosis programs and induce apoptosis [6]. Apoptosis of a large number of immune cells and non-immune cells during the occurrence and development of sepsis is a key factor causing death in sepsis patients [10]. Chang et al. [11] studied and found that the number of apoptotic cells in peripheral blood of sepsis patients is markedly larger than that in non-sepsis patients, which is positively associated with organ failure in sepsis patients. Caspase3 is the major intracellular apoptosis protein that links apoptosis via the mitochondrial pathway and death receptor pathway, which is the key to initiation of apoptosis [12]. In addition, the Bcl-2 family proteins are a group of key proteins regulating apoptosis in the cytoplasm, among which Bax can promote apoptosis and Bcl-2 can suppress apoptosis, playing crucial roles in lymphocyte apoptosis [13]. In this study, the results manifested that sepsis could markedly raise the lymphocyte apoptosis level in peripheral blood, remarkably increase the expression of Caspase3 in lymphocytes and reduce Bcl-2/Bax. During oxidative stress, a large amount of ROS will be produced, and the body tissues will be damaged if the ROS content is beyond the body's antioxidant defense ability. Jiang et al. [14] showed that a large amount of ROS in cells can interact with such intracellular macromolecules as proteins, lipids and nucleic acids, induce intracellular mitochondrial apoptosis and damage the biological membrane, causing cell death. In this study, the content of ROS in lymphocytes in sepsis mice had a significant increase.

FoxO factor is a highly conserved subfamily, and FoxO3a, as an important transcription factor in the FoxO family, is involved in such processes as oxidative stress, apoptosis and proliferation of cells, and regulates the stability of intracellular environment through interaction with other signaling pathways [15-17]. Huang et al. [18] studied and found that FoxO3a maintains biological activity in the nucleus, and the increased phosphorylation level can promote nuclear exclusion of FoxO3a and localization in the cytoplasm, resulting in the loss of transcriptional activity. In this study, it was found that the level of FoxO3a in lymphocytes was decreased, the phosphorylation level of FoxO3a was increased, the levels of p-PI3K and p-Akt rose, and lymphocyte apoptosis was enhanced in sepsis mice. The study of Lu et al. [19] showed that FoxO3a can mediate the occurrence of inflammatory response in vivo, activate the expression of FoxO3a in myocardial cells, and significantly reduce the production of inflammatory factors. In this study, after FoxO3a was activated by IturinA, the content of IL-1β was decreased and that of IL-10 was increased in cells, lymphocyte apoptosis was weakened, and the content of ROS declined.

5. Conclusions

In conclusion, sepsis can inhibit the activity of FoxO3a in cells, increase the level of p-FoxO3a and the content of ROS and inflammatory factors in lymphocytes, and lead to lymphocyte apoptosis, thereby causing death of sepsis mice. Activating FoxO3a in lymphocytes can remarkably reduce the mortality rate of sepsis mice.

Conflict of Interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for

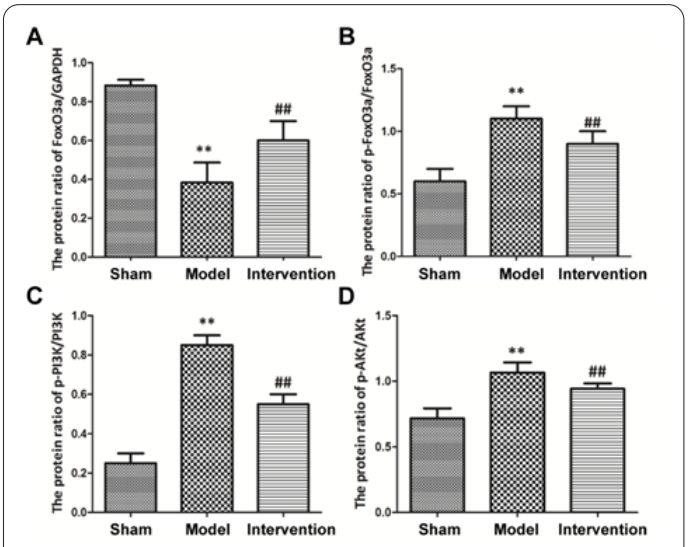

Fig. 6. Expression levels of FoxO3a signaling pathway-related proteins. Compared with sham group and intervention group, model group has significantly lower expression of FoxO3a, and significantly higher expressions of p-FoxO3a, p-PI3K and p-Akt. **P<0.01 *vs.* sham group, ##P<0.01 *vs.* model group.

publication.

Ethics approval and consent to participate
This study was approved by the Animal Ethics Committee of Shanxi Medical University Animal Center.

Informed Consent
The authors declare not used any patients in this research.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions
Ya Wen designed the study and performed the experiments, Yan Li collected the data, Jinli Guo and Kaijian Shang analyzed the data, Ya Wen prepared the manuscript. All authors read and approved the final manuscript.

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