

Role of Thrombospondin-1 in Sepsis-Induced Myocardial Injury

YUN XIE

shanghai general hospital

JIAXIANG ZHANG

Shanghai General Hospital

WEI JIN

shanghai general hospital

RUI TIAN

shanghai general hospital

Ruilan Wang (✉ wangruilan0@163.com)

shanghai general hospital

Research Article

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Abstract

Objective: Sepsis often causes myocardial injury with a high mortality. We wanted to investigate the effects of thrombospondin-1 (THBS1) expression on myocardial cell injury, oxidative stress and apoptosis in sepsis.

Methods: The expression of THBS1 mRNA in LPS-induced mouse primary cardiomyocytes was detected by real-time fluorescence quantitative PCR. We constructed a eukaryotic siRNA expression vector and used liposome transfection to knockdown THBS1 mRNA expression in myocardial cells. We detected the THBS1 mRNA expression level using real-time fluorescent quantitative PCR. Four groups were used: control, LPS, THBS1 siRNA, and LPS + THBS1 siRNA. ELISA was used to detect cTnI, proBNP, ROS, caspase3 and other indicators of cell damage. At the same time, sepsis mouse models were prepared for H&E, TUNEL and caspase-3 staining to evaluate myocardial cell injury and apoptosis. Clinical samples were collected to analyze the serum THBS1 level and correlate it with the prognosis of patients with myocardial injury of sepsis.

Results: The expression level of THBS1 mRNA in myocardial cells induced by LPS was increased, and the serum THBS1 level in patients with myocardial injury in sepsis was also significantly increased. In the THBS1 siRNA group with myocardial injury, the levels of cTnI and proBNP were significantly decreased, the levels of the inflammatory cytokines IL-6 and TNF- α were significantly decreased, ROS were significantly decreased, and caspase3 was significantly decreased, and myocardial cell apoptosis was also reduced in the sepsis mouse model.

Conclusion: THBS1 is closely related to the biological behavior of myocardial cells and may be a therapeutic target for myocardial injury in sepsis.

1 Introduction

Sepsis is a life-threatening organ dysfunction caused by the host's dysfunctional response to infection, with a dangerous condition, high morbidity and mortality [1]. Myocardial injury is one of the most common complications of sepsis, which mostly occurs in the middle and late stages of the disease [2]. Studies have shown that nearly 50% of sepsis patients are complicated with myocardial depression, which is manifested by left and right ventricular cardiac dysfunction and reduced left ventricular ejection fraction, and the mortality rate is as high as 70% [3]. Cardiac dysfunction may complicate the course of sepsis and septic shock, and cardiac dysfunction caused by myocardial injury is one of the important causes of death in patients with sepsis [3,4]. At present, the specific mechanism of septic cardiomyopathy is not clear, and the activation of apoptosis pathway plays an important role in septic myocardial injury [5]. Studies have confirmed that different activation of Caspases and release of mitochondrial cytochrome C can be found in septic cardiomyocytes [6]. Inhibition of cell apoptosis can reverse the occurrence of septic myocardial injury [5]. Maintaining myocardial mitochondrial membrane potential and inhibiting the activation of apoptotic proteins Caspase 3 and 7 can reduce the apoptosis of

myocardial cells [7]. Therefore, how to improve the myocardial injury in sepsis and reduce the apoptosis of myocardial cells is our research direction.

Thrombospondin-1 (THBSs or TSP) is a secretory glycoprotein involved in various functional areas of embryonic development, wound healing, angiogenesis, and inflammatory reactivity [8, 9]. THBS has a multimodular structure, with each region performing different functions by specific binding of different factors, and high-resolution determination shows its unique and interesting protein structure. THBSs are divided into two subgroups: A and B. Group A includes THBS1 and THBS2, which can form trimers. Group B includes THBS3, THBS4 and THBS5, which can form pentamers [10]. According to the THBS molecular structure they can be divided into two groups, the THBS1 type and the THBS2 α repetitive sequence TSRs (group A), and the other members do not contain this sequence (group B) [13]. TSRs, also known as lysine repeat sequences, are involved in cell attachment, inhibition of angiogenesis, protein-protein and protein-mucopolysaccharide interactions [14]. Therefore, THBS1 can regulate the adhesion, migration, proliferation and neovascularization of vascular endothelial cells. Group B members lack the TSRs sequence and therefore have no antiangiogenic effect in the tumor microenvironment [12].

Among the THBS family, THBS1, with a relative molecular weight of 450×10^3 , can regulate tumor growth, cell migration and vascular formation [11]. Research on THBS1 in septic cardiomyocytes has rarely been reported. We transfected THBS1 small interfering RNA (siRNA) into cardiac cell lines with relatively high THBS1 protein expression through liposomes, silencing the THBS1 gene to reduce THBS1 expression in cardiac cells, and observed the effects of THBS1 gene silencing on myocardial cell injury, oxidative stress, the inflammatory response and apoptosis.

There are some researchers studied the mechanism of organ damage induced by sepsis—especially myocardial injury [41], however, the specific effects of thrombospondin-1 (THBS1) expression on myocardial cell injury, oxidative stress and apoptosis in sepsis have not been entirely understood. This study focused on the relationship between THBS1 and sepsis-induced myocardial injury, and studied the oxidative stress, the inflammatory response, apoptosis and its molecular mechanism from the perspective of THBS1.

2 Materials And Methods

2.1 Animals, cells and main reagents

Male C57dx newborn mice (3 days old, SLAC, Shanghai, China) and male C57BL/6 mice (8 weeks old, SLAC, Shanghai, China), were purchased from the experimental animal center of Shanghai Jiao Tong University. RPMI 1640 medium and fetal bovine serum were purchased from Gibco, USA. LPS and dimethyl sulfoxide (DMSO) were purchased from Sigma; the diquinolinic acid (BCA) protein quantitative kit was purchased from the Nanjing Nuowizan company; THBS1 siRNA and control siRNA were purchased from Santa Cruz, USA. THBS1 mouse anti-human monoclonal antibody (abl823) was purchased from Abcam. Anti-actin and sheep anti-mouse polyclonal antibodies were purchased from the

Beijing Zhongshan Jinqiao Biotechnology company. All experimental procedures were carried out according with the guidelines of the Institutional Animal Care and Use Committee of Shanghai. All of the experimental procedures were performed after obtaining the approval of the Ethical Committee for Animal Experiments of Shanghai general hospital (Shanghai, China).

2.2 Cell culture and stimulation

We randomly selected 12 C57dx 3d old mice. After cervical dislocation, their hearts were extracted, the atria were cut off, the hearts were rinsed with LML collagenase, and the heart tissue was cut into approximately 1 mm³ pieces. The pieces of heart tissue were mixed with collagenase and were transferred into a centrifuge tube and were repeatedly shaken and mixed. After allowing it to stand and any solid tissue had settled, the supernatant was removed and this was repeated until the tissue mass was dissolved completely. The obtained supernatant was centrifuged at 2000 r/min for 10 min, discarded, and the precipitate was mixed with 2 ml of medium, and then inoculated into a 75 ml culture bottle. After incubating at 37°C for 90 min, the nonmyocardial cells had adhered to the bottle, and the cell suspension, which contained the myocardial primary cells, was inoculated into another culture bottle.

2.3 Detection of THBS1 mRNA in myocardial cells was performed by real-time fluorescence quantitative PCR (qRT-PCR). Total RNA was extracted from the cardiomyocytes according to the instructions of the TRIzol reagent. The concentration of RNA was measured by a micro ultraviolet spectrophotometer and its purity was determined. The RNA was reversely transcribed into cDNA using a reverse transcription kit and amplified according to the instructions of the qRT-PCR kit. After the reaction, the amplification curve and dissolution curve were confirmed, and the results were interpreted strictly in accordance with the instructions of the kit. The $2^{-\Delta\Delta Ct}$ method was used to calculate the level of THBS1 mRNA expression.

2.4 Western blotting analyses

The cells were placed in the cell lysis solution. The total protein concentration was detected using a BCA kit. The protein samples (60 µg per well) were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and then transferred to a PVDF membrane. The membrane was blocked and then incubated with the indicated primary antibody at 4 °C overnight, washed, then incubated with the secondary antibody. The signal was detected with an ECL system using an A1600 detector and photographic exposure system. Protein expression levels were calculated using actin as the internal reference.

2.5 Effects of transfected siRNA on myocardial cell injury, oxidative stress, inflammatory response and apoptosis

2.5. 1 In the experimental group, primary cells were inoculated into a 25 cm² culture flask and RPMI 1640 medium containing 10% fetal bovine serum by volume was added. After the cells grew well, they were digested with trypsin and inoculated into 6-well plates. Four groups were set up for the experiment: A. Control group; B. LPS group; C. THBS1 siRNA group; and D. LPS+THBS1 siRNA group. There was no

intervention in the control group; in the LPS group, we used 1 mg/L LPS to stimulate the cardiomyocytes. THBS1 siRNA group: THBS1 siRNA was transfected into the cardiomyocytes with Liposome 2000. LPS+THBS1 inhibition group: THBS1 siRNA was transfected with Liposome 2000 into LPS-treated cardiomyocytes.

2.5. 2 Detection of THBS1 mRNA in cells after transfection for 48 h. The expression of THBS1 mRNA in the cells was detected by qRT-PCR, using the same method as 1.2. Each group was tested with three biological replicates twice.

2.5. 3 Cell injury, oxidative stress, inflammatory response and apoptosis were detected 48 h after transfection. The cells were inoculated into a 96-well plate. After routine culture for 24, 48, 72 and 96 h, expression of cTNI, proBNP, ROS, IL-6, TNF- α and caspase-3 were detected by ELISA.

2.6 Sepsis model:

Healthy SPF C57BL/6 mice(n=18), male, 8 weeks old, weight approximately 20 ± 2 g, were used. Before the experiment, the mice were given free food and water and kept at an ambient temperature of 20°C. Intraperitoneal injection of LPS 15 mg/kg was used to make the model. To judge the success of establishing model according to the references[42], It was confirmed that the sepsis modelling was successful by histopathological changes in cardiac muscle 24 h after modelling and cTNI, proBNP, IL-6, and TNF- α expression by ELISA. Three groups(n=6 every group) have been designed in the animal experiments. A. Control group; B. LPS group; C. LPS+THBS1 Inhibition group.

2.7 H&E, TUNEL and caspase-3 staining

H&E staining and TUNEL analysis were performed to assess the histopathological changes in myocardial tissue. Solarbo (4% paraformaldehyde) was used to fix the mouse myocardium overnight at 4°C. Paraffin embedding, tissue sections and H&E staining were performed as described above. A TUNEL assay kit (Roche Diagnostics, Indianapolis, IN, USA) was used to measure the rate of apoptosis of the cardiomyocytes, according to the manufacturer's specifications. A caspase-3 fluorometry kit (Beyotime Institute of Biotechnology, Shanghai, China) was used for detection. We did not test LDH activity, which is a marker of cell injury ,Because LDH is greatly affected by Sepsis[43].

2.8 Collection of clinical samples. Serum was collected from patients with myocardial injury of sepsis, those without myocardial injury of sepsis and healthy people and stored at -80°C. THBS1 was detected by ELISA. The prognosis of the patients was collected during the follow-up of 28 days.

2.9 Statistical analysis. SPSS 17.0 was used to process the data. The expression levels of THBS1 mRNA, cTNI, proBNP, ROS, IL-6, TNF- α and caspase-3 in the different groups were compared by one-way ANOVA. LSD-t test was used for pairwise comparisons. Statistical significance was defined as $p = 0.05$.

3 Results

3.1 THBS1 expression by qPCR and western blot

In the LPS-stimulated primary cardiomyocytes, qPCR showed a significant increase in THBS1, and western blot also showed a significant increase in THBS1 (Figure 1A, 1B).

3.2 caspase3 and ROS expression by ELISA

In LPS-stimulated primary cardiomyocytes, intracellular ELISA showed a significant increase in caspase3 and ROS expression in the LPS group. After the reduction of THBS1 through siRNA, ROS were significantly decreased, the oxidative stress response was decreased, caspase-3 was significantly decreased, and myocardial cell apoptosis was decreased (Figure 1C, 1D).

3.3 cTNI, proBNP, Il-6, and TNF- α expression by ELISA

In the LPS-stimulated primary myocardial cells, ELISA showed that the cTNI, proBNP, Il-6, and TNF- α levels in the LPS group were significantly increased. After the reduction of THBS1 caused by siRNA, cTNI, proBNP, IL-6 and TNF- α levels were significantly reduced (Figure 2).

3.4 Histopathological changes in cardiac muscle 24 h after modelling

In the mouse cardiac pathology sections from mice intraperitoneally injected with LPS at 15 mg/kg, myocardial histopathological changes were observed 24 h after mouse modeling under a light microscope:

In the control group (Figure 3 A, D), the myocardial fibers were arranged neatly with clear horizontal stripes. The cardiomyocytes showed normal morphology, the cytoplasm was pink, the nuclear membrane was intact, the chromatin was dense and deeply stained, and there were no significant changes in the myocardial interstitium.

LPS (LPS 15 mg/kg group, Figure 3 B, E) the myocardial fibers were irregular, there was interstitial edema, hemorrhage, and inflammatory cell infiltration.

LPS+THBS1 inhibition group (Figure 3C and F). There was interstitial edema and bleeding, but inflammatory cell infiltration was reduced.

3.5 TUNEL analysis and caspase3 fluorescence for myocardial injury in sepsis mice

TUNEL staining was used to observe the changes of the myocardial cells in mice 24 h after modeling: LPS (LPS 15 mg/kg group, Figure 4B) showed increased apoptosis compared with the normal control group (control heart group, Figure 4A), while the LPS+THBS1 inhibition group (Figure 4C) showed decreased apoptosis.

3.6 Caspase-3 staining

Caspase-3 staining was used to observe the changes of myocardial cells in mice 24 h after modeling:

The LPS 15 mg/kg group (Figure 4E) showed increased caspase3 compared with the control heart group (Figure 4D), and there was decreased caspase3 in the LPS+THBS1 inhibition group (Figure 4F).

3.7 Characterization of THBS1 expression in patients with sepsis myocardial injury in clinical research

In the clinical samples, THBS1 was significantly increased in patients with sepsis accompanied by myocardial injury, and THBS1 was also higher in deceased patients than in those who survived. The survival time of the high THBS1 group was lower than that of the low THBS1 group for predicted 28-day mortality. THBS1 predicted myocardial injury (AUROC=0.817, $p < 0.001$) (Figure 5).

4 Discussion

Although THBS1 has been shown to have positive effect on various biological functions, the benefits of THBS1 for the cure of cardiac injury are unclear. THBS1 promoted the over production of free radicals and ROS [44]. Free radicals attack the cell membrane, increase cell death, and result in the production of caspase-3. Our results has shown that decrease of THBS1 effectively reduces caspase-3 levels, and decreases ROS concentration and cTnl activity after LPS treatment. Furthermore, in this study, decrease of THBS1 attenuates The viability inhibition LPS induced in myocardial cells. These results indicate that decrease of THBS 1mitigates the oxidative damage in LPS-induced cardiac injury. It is worth mentioning that the metabolic pathways of THBS1 in body maybe partly related to the form of THBS1 species. However, there is no evidence that THBS1 may react directly with AKT1, so whether THBS1 takes part in this process still needs to be furtherly investigated.

Apoptosis, as a kind of programmed cell death, can be triggered by much environmental or chemical irritant, such as ROS [45–47]. Subsequently, excessive cytochromec release, and activates caspase-3 resulting in cleaved caspase-3[48]. We have shown that decrease of THBS1 decreases the levels of caspase-3. Moreover, TUNEL assay results confirm that the myocardial apoptosis induced by LPS is significantly increased by THBS1. Hence, the results suggest that THBS1 promotes LPS-induced myocardial apoptosis.

As a potent inflammatory mediator, TNF- α can be activated by oxidative stress [49]. Under the stimulation of pro-inflammatory cytokines, TNF- α is activated, then accumulates and accelerates the production of IL-6 which is a pro-inflammatory cytokines. Due to the adverse impact of oxidative stress, the cell upregulates genes involved in inflammation to activate or amplify the inflammatory response [46]. Our results show that TNF- α and the increase in IL-6 induced by LPS are substantially suppressed by decreasing THBS1. Thereby, Decrease of THBS1 may protect against LPS-induced cardiac injury by reducing the expression of inflammatory cytokines to weaken the inflammatory response.

Studies on THBS1 in human sepsis are extremely limited, with a few studies showing no clear correlation between THBS1 expression and the prognosis of sepsis [16], and a single-center cohort study in intensive care showed no correlation between baseline platelet reactive protein 1 concentration and mortality in

patients with sepsis [16]. However, in our study, THBS1 was found to be able to predict myocardial damage. This is worthy of further multicenter large sample confirmation.

Individual studies have confirmed that THBS1 may be associated with sepsis immunity [17], and platelet reactive protein 1 leads to the death of septic mice through its effect on innate immunity [17]. Regarding inflammation, studies have shown that the production of platelet reactive protein 1 regulates the secretion of inflammatory cytokines in THP-1 cells through the NF- κ B signaling pathway [31]. Studies have shown that gingiva stem cells can improve lipopolysaccharide induced inflammation by secreting TGF- β 3 and THBS1, which can induce M2-polarization of macrophages [32]. In our study, lowering THBS1 led to reduced inflammation, reduced oxidative stress, reduced myocardial injury, and reduced apoptosis of cardiomyocytes. The specific mechanism still needs further study.

We used qRT-PCR to show that the expression level of THBS1 mRNA in LPS-induced primary cardiomyocytes was higher than that in normal cardiomyocytes. This study also confirmed in clinical samples that the expression level of THBS1 in patients with myocardial injury of sepsis was also higher than that in the normal control group. We constructed siRNA and transfected it into the primary cardiomyocytes with the highest THBS1 expression. THBS1 mRNA expression in the cells was significantly downregulated, while downregulated THBS1 expression in the cells could inhibit oxidative stress of cardiomyocytes and inhibit their apoptosis.

The novelty of manuscript was that we found the effects of thrombospondin-1 (THBS1) expression on myocardial cell injury, oxidative stress and apoptosis in sepsis.

5 Conclusion

The results of this study suggest that THBS1 may be closely related to the occurrence and development of myocardial injury in sepsis, which lays a foundation for research into THBS1 as a therapeutic target for myocardial injury in sepsis.

Declarations

Acknowledgements

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Authors' contributions

YX, JXZ, RT, WJ, JH and RW contributed to the study conception and design, analysis and interpretation of data; drafting of the article and critical revision for important intellectual content. All of the authors have read and approved the final manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Patient consent for publication

Not applicable.

Authors' information (optional)

Department of Critical Care Medicine, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, 650 New Songjiang Road, Songjiang, Shanghai, 201600, China.

Competing interests

The authors declare that they have no competing interests.

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Figures

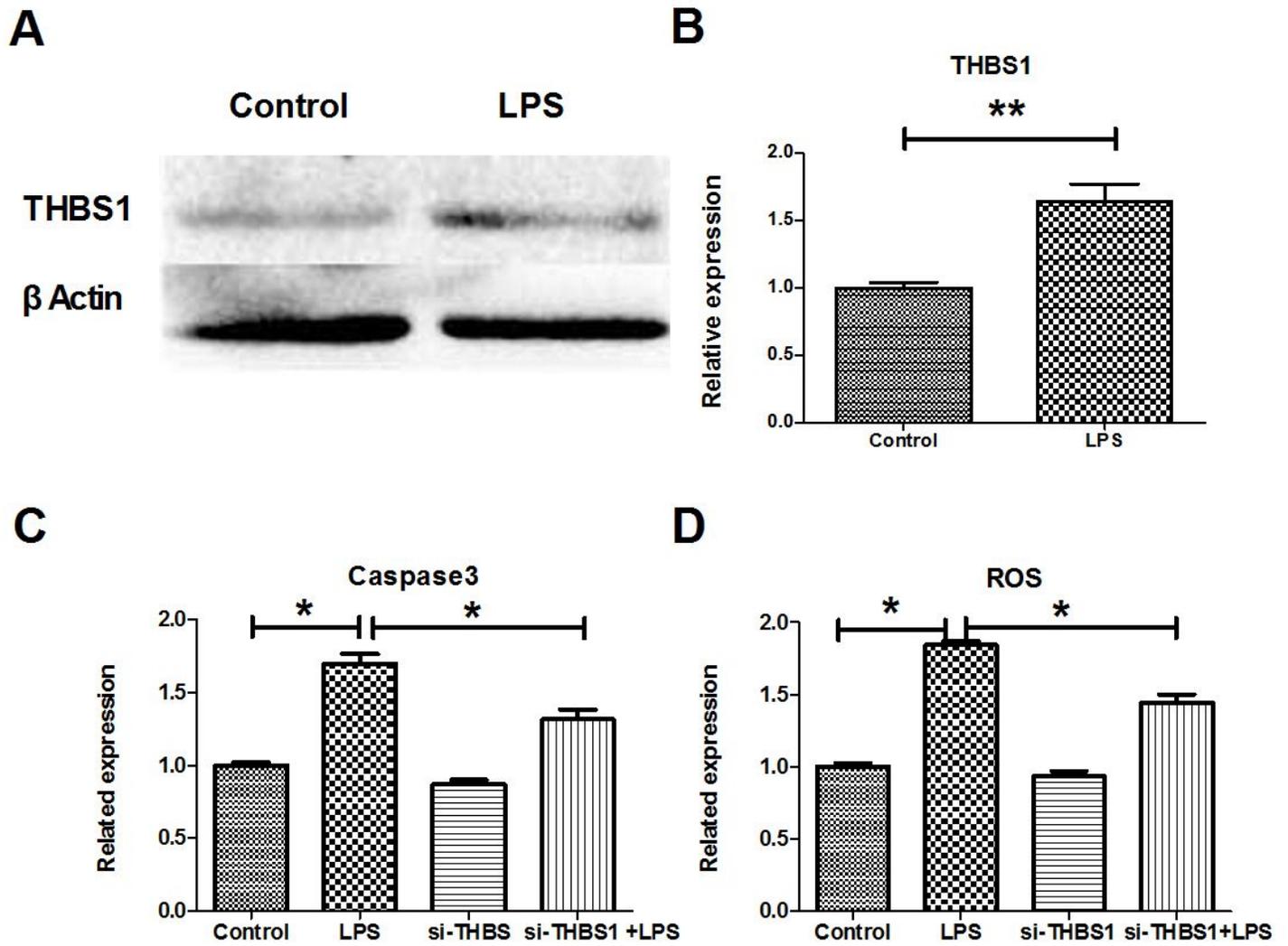


Figure 1

Expression of THBS1, caspase3 and ROS in LPS-stimulated primary cardiomyocytes (A. Western blot of THBS1, B. qPCR of THBS1, C. ELISA of caspase3, D. ELISA of ROS)

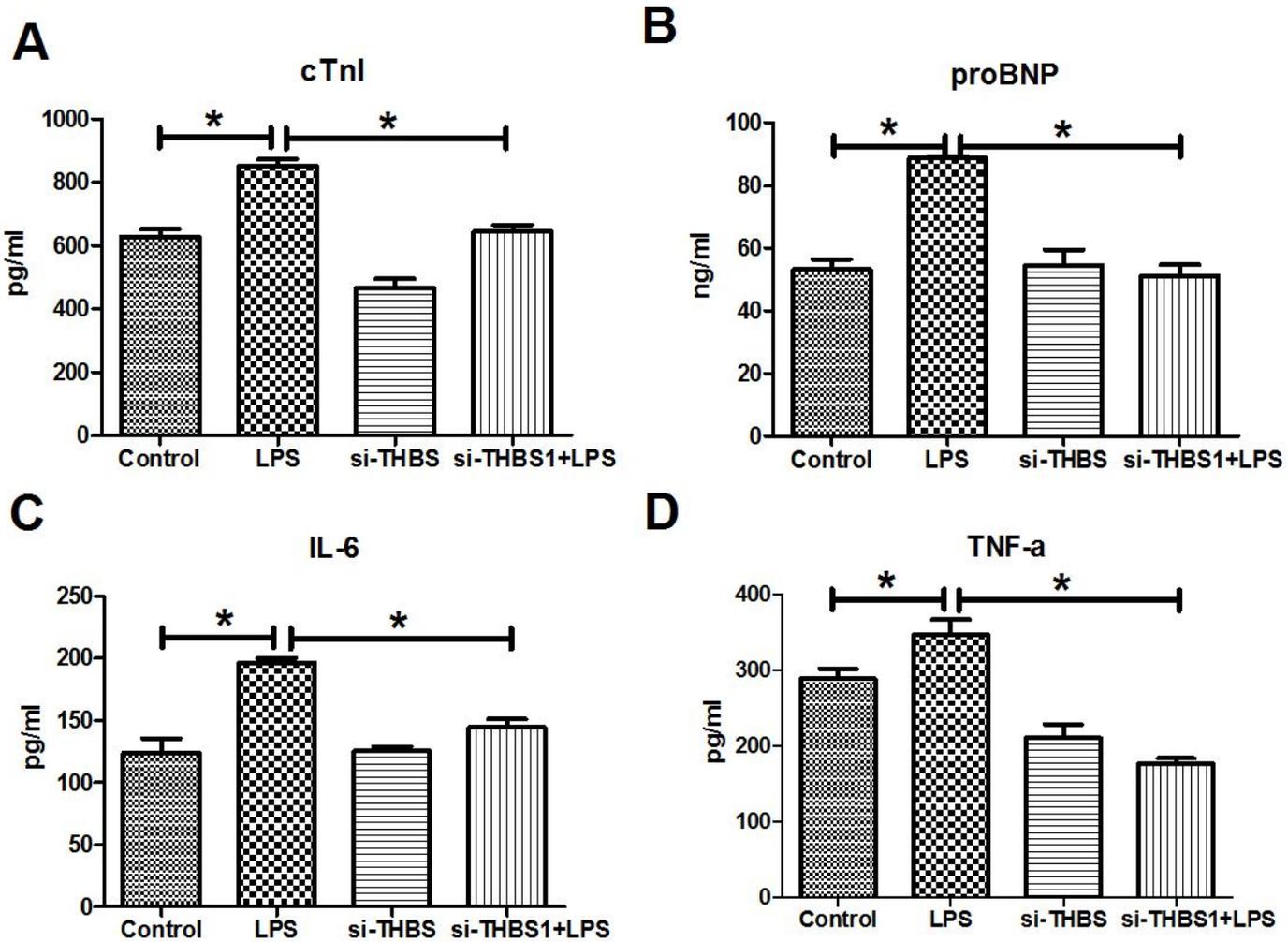


Figure 2

Expression of cTNI, proBNP, IL-6, and TNF-α in the LPS-stimulated primary cardiomyocytes(A. cTNI, B.proBNP, C.IL-6, D. TNF-α)

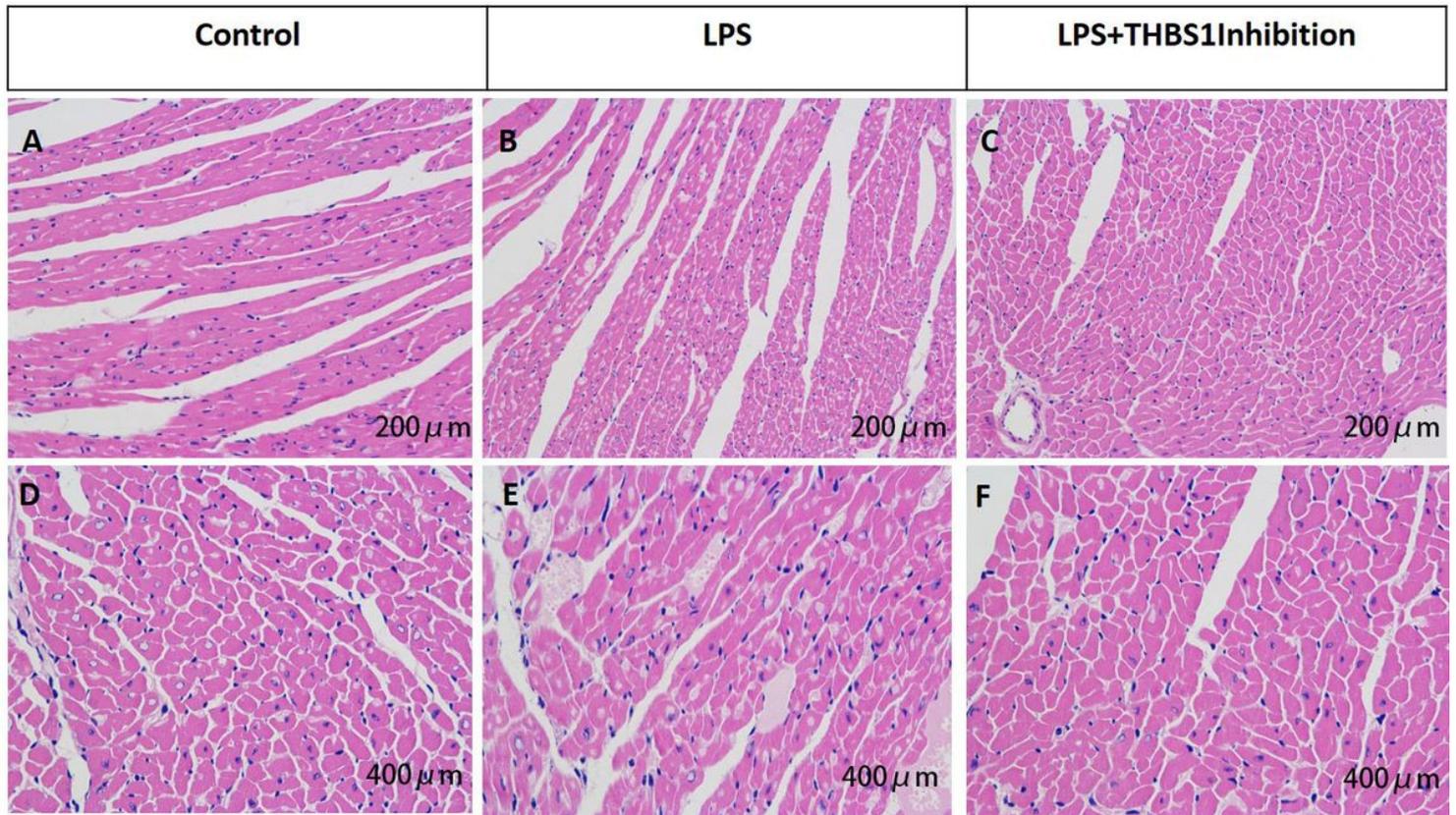


Figure 3

Histopathological changes in cardiac muscle 24 h after modelling

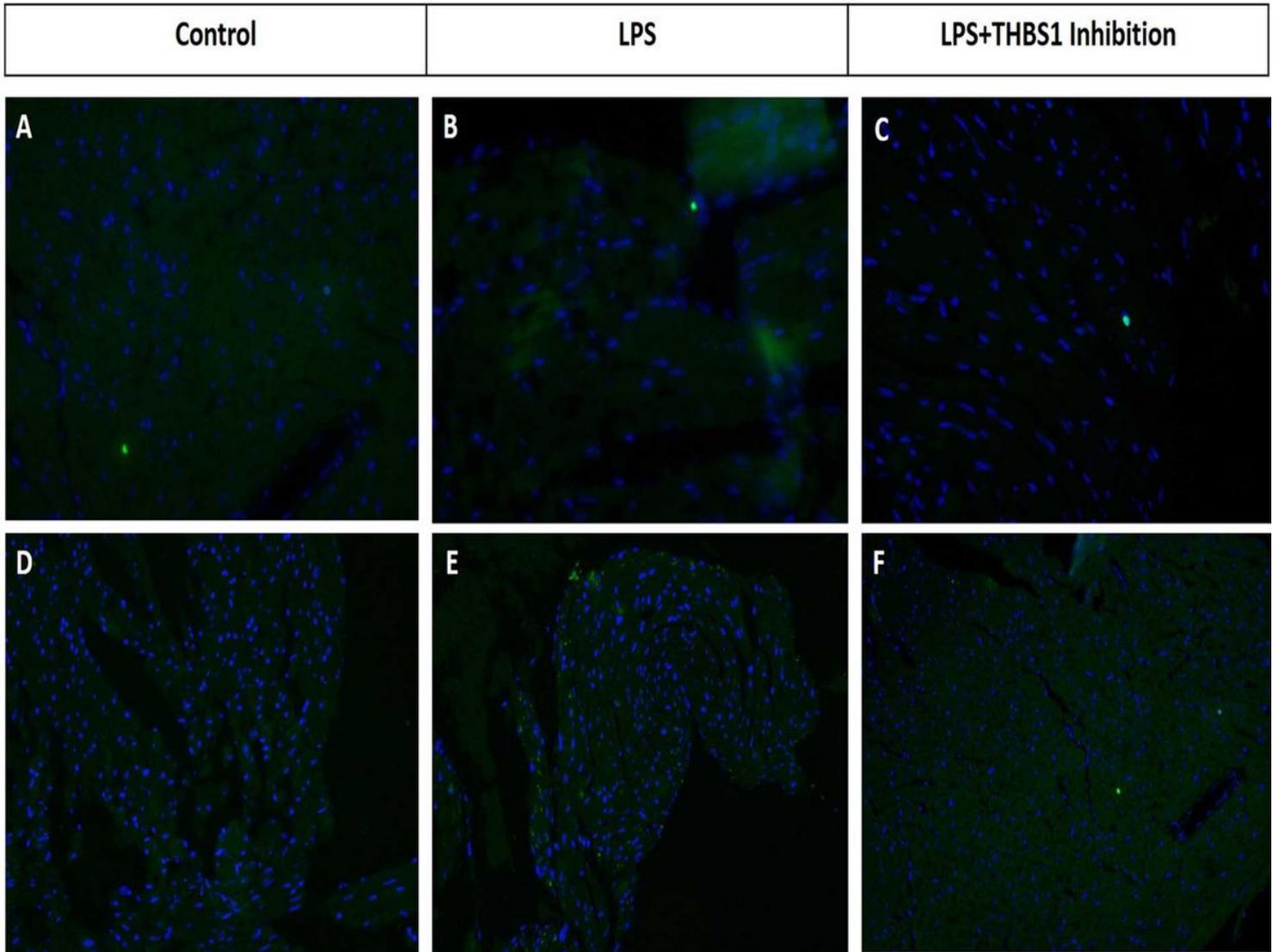


Figure 4

TUNEL analysis and caspase3 fluorescence for myocardial injury in sepsis mice

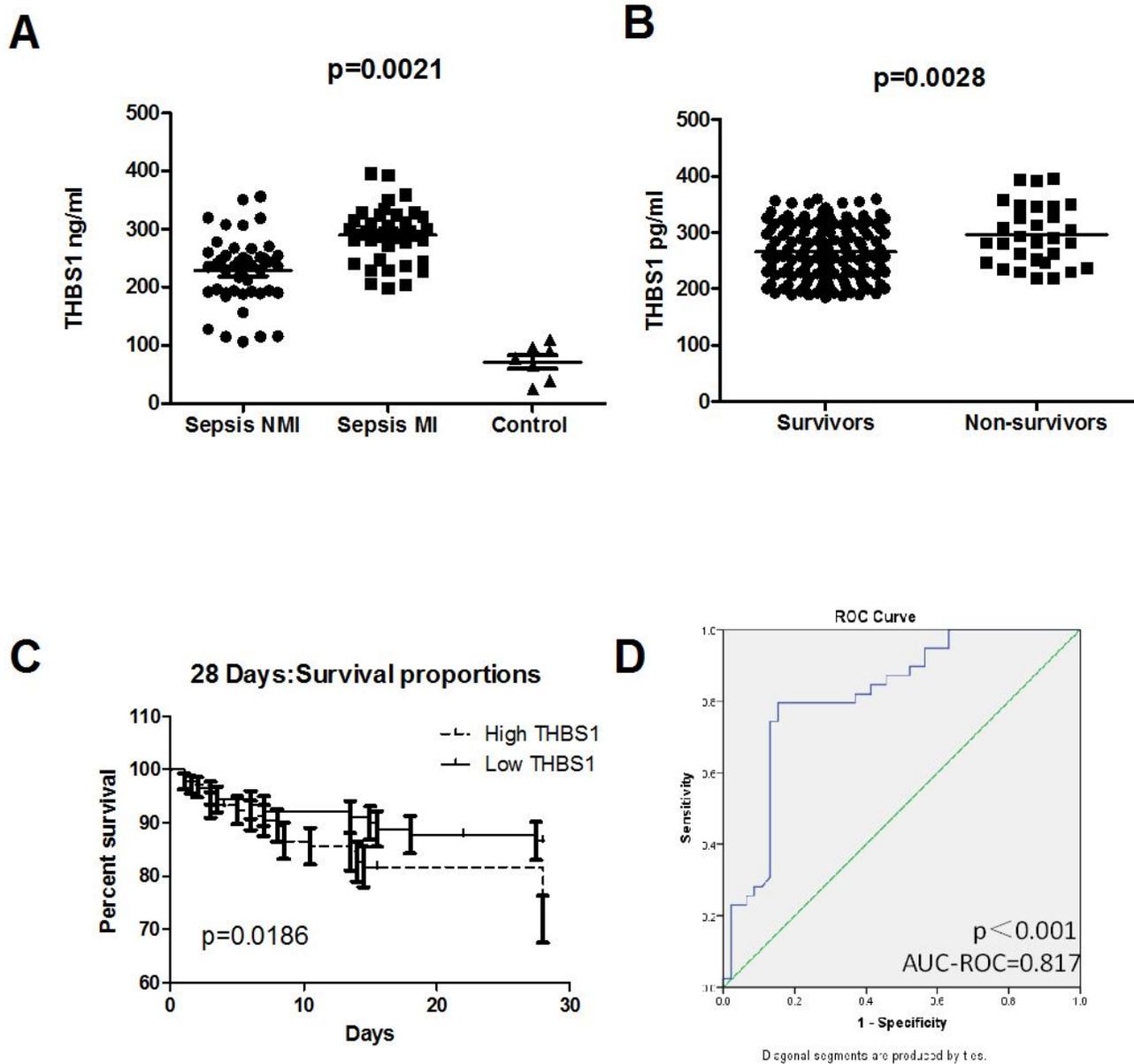


Figure 5

Characterization of THBS1 expression in patients with sepsis myocardial injury in clinical research (A. Expression of THBS1 in patients with sepsis, sepsis accompanied by myocardial injury, B. Expression of THBS1 in survivors and non-survivors. C. Survival curve of the high THBS1 group and the low THBS1 group. D. ROC curve of THBS1 in predicting myocardial injury.