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Effects of ubiquitin-proteasome inhibitor on the expression levels of TNF-α and TGF-β1 in mice with viral myocarditis

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Abstract. Effects of ubiquitin-proteasome system (UPS) inhibitor MG-132 on the expression levels of tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β1) in mice with viral myocarditis were investigated to analyze the correlation of myocardial tissue score of mice between TNF-α and TGF-β1. Eighty healthy male SPF mice aged 6 weeks were selected and 20 mice were randomly selected as the blank group. The blank group did not receive any intervention. Mortality rates of each group were recorded and compared on day 8 of modeling, and heart specimens from the remaining mice were histopathologically examined and the expression of mRNA and protein of TNF-α and TGF-β1 in myocardial tissues were detected by western blot analysis. Correlation between mouse myocardial histopathologic scores and expression of protein of TNF-α and TGF-β1 in myocardial tissues, as well as the expression of TNF-α and TGF-β1 in myocardial tissue in VMC mice was analyzed. The expression levels of myocardial histopathological scores, mRNA and protein of TNF-α and TGF-β1 in the blank and control group were significantly lower than those in the VMC and the MG-132 group. The myocardial histopathological scores, mRNA and TNF-α and TGF-β1 protein in the MG-132 group were significantly lower than those in the VMC group ($P<0.05$). The expression of TNF-α and TGF-β1 protein in myocardial tissues was positively correlated with the pathological score in myocardial tissue of mice ($r=0.843$, $P<0.05$; $r=0.763$, $P<0.05$), and there was a positive correlation between the expression of TNF-α and TGF-β1 protein in myocardial tissues of VMC mice ($r=0.672$, $P<0.05$). UPS inhibitor MG-132, which can significantly alleviate the myocardial injury of VMC mice, reduced the expression of inflammatory factors in myocardial tissues, and improved the survival rate of mice, thus it is a potential new treatment for VMC.

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Key words: ubiquitin-proteasome inhibitor MG-132 and CVB3, viral myocarditis, tumor necrosis factor α, transforming growth factor-β1, myocardial histopathological scores

Introduction

Viral myocarditis (VMC), as one of the most common cardiac infectious diseases, is mainly caused by myocardial cells infected with the virus, causing localized or diffuse inflammation of the myocardium. The virus causes direct damage to the myocardial cells and stimulates the immune response, thus causing sustained damage to the myocardial tissues (1,2). Coxsackie B3 virus (CVB3) is the most important virus that causes myocarditis. CVB3 can induce oxidative stress response and apoptosis in a few weeks in the pathogenesis of VMC, resulting in arrhythmia, myocardial failure and may eventually lead to sudden death, but special treatment of VMC has not been found as yet (3,4).

Ubiquitin-proteasome system (UPS), as an important ATP-dependent protein control system in eukaryotic cells, not only participates in physiological processes such as apoptosis, inflammatory response and intracellular signaling, but also plays an important role in maintaining cell homeostasis (5-7). In recent years, studies have found that UPS not only plays an important role in the inflammatory response of various diseases, but also plays a key role in the occurrence and development of various viral infectious diseases (8,9). As an aldehyde peptide proteasome inhibitor that can inhibit the activity of chyme protein, MG-132 also has a protective effect on the occurrence of inflammatory reactions in many diseases (10). As a cytokine with a variety of biological effects produced by macrophages, tumor necrosis factor-α (TNF-α) has been shown to play an important role in the inflammatory response of VMC (11). However, transforming growth factor-β1 (TGF-β1) is an initiating factor synthesized from the extracellular matrix of collagen fibers, and it is also one of the many factors leading to the occurrence of viral myocarditis (11).

At present, few studies have reported the role of UPS in the inflammatory reaction of VMC, so we explored the effect of ubiquitin-proteasome inhibitor MG-23 on the expression levels of serum TNF-α and TGF-β1 in CVB mice, in order to understand the inflammatory mechanism of VMC.

Table I. Sequences of the primers.

Factor	Upstream primer	Downstream primer
TNF-α	5'-CCACGCTCTTCTGTCTACTGA-3'	5'-AAGGTACAACCCATCGGCTG-3'
TGF-β1	5'-CCAACTATTGCTTCAGCTCCA-3'	5'-GTGTCCAGGCTCCAA ATGT-3'
GAPDH	5'-GGTTGTCTCCTGCGACTTCA-3'	5'-TGGTCCAGGGTTTCTTACTCC-3'

TNF-α, tumor necrosis factor-α; TGF-β1, transforming growth factor-β1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Materials and methods

Experimental animals and materials. A total of 80 healthy male SPF grade mice aged 6 weeks and weighing 25.1±5.53 g were selected. All the mice were purchased from the animal experiment center of Zhejiang province, with the production license of SCXK (Zhejiang) 2011-0166. The mice were fed in a plastic box with bedding material on a solid bottom with the temperature of 22˚C and the relative humidity between 50 and 65%, 12 h day-night rhythm was normal, and they were free to eat and drink.

The study was approved by the Ethics Committee of Central Hospital of Zibo (Zibo, China). The CVB3 virus was purchased from Cell Signaling Inc. at a titer of 100 TCID50 (50% tissue culture infective dose) /0.1 ml. Mg-132 was purchased from Calbiochem Inc. at a concentration of 0.75 mg/kg.

Grouping and modeling. Twenty mice were randomly selected as the blank group, and they were kept in routine feeding without any intervention. The remaining 60 mice were then randomly divided into the VMC, MG-132 and control group, each containing 20 mice. Mice in the control group were intraperitoneally injected with 0.1 ml PBS (phosphate buffer-saline) at 0.1 mmol/l, mice in the VMC and MG-132 group were intraperitoneally injected with 0.1 ml diluent of CVB3 at 100 TCID50/0.1 ml, and mice in the MG-132 group were intraperitoneally injected with 0.75 mg/kg MG-132 the day after the injection of the CVB3 virus. They were continuously injected for 7 days. Mortality rates of each group were recorded and compared on day 8 of modeling, and then peripheral blood and heart samples of the remaining mice were taken for subsequent detection.

Pathological examination. Hearts of mice were fixed with 10% formaldehyde and dehydrated routinely, then embedded with paraffin and sectioned. After sectioning, the pathological changes of mouse myocardial tissues were observed under light microscope (Olympus Corp.) and the pathological score of the myocardial tissues was evaluated. The judgement scores were as follows: 1 point, when the proportion of inflammatory cell infiltration and myocardial necrosis was <25%, 2 points, when the proportion of inflammatory cell infiltration and myocardial necrosis was between 25 and 50%, 3 points, when the proportion of inflammatory cell infiltration and myocardial necrosis was between 51 and 75%, 4 points, when the proportion of inflammatory cell infiltration and myocardial necrosis was >75%.

Expression levels of mRNA in TNF-α and TGF-β1 detected by RT-qPCR. First, the TRIzol reagent (purchased from Applide Invitrogen, Inc.) was added into the myocardial tissue of mice to extract the total RNA in the blood, and the concentration and quality of total RNA were detected by ultraviolet spectrophotometer (purchased from Shanghai Yuanxi Instrument Co., Ltd.). Total RNA (2 μl) was used to compound cDNA in strict accordance with the instructions of minScript reverse transcription kit (Takara Bio, Inc.). Synthetic cDNA (2 μl) was used for qPCR (RT-qPCR kit was purchased from Takara). Total RNA (2 μl) was added to a microcentrifuge tube, then 11 μl of DEPC H_2O was added. A total of 12-18 μl of 10 μM Oligo (dT) was added, mixed and heated at 70˚C for 10 min. After ice bath for 1 min, 2 μl 10X PCR buffer, 2 μl 25 mM $MgCl_2$, 1 μl 10 mM dNTP mix and 2 μl 0.1 M DTT were added, mixed and incubated for 3 min at 42˚C. A total of 1 μl of Superscript II was added, incubated at 42˚C for 30 min, and heated at 70˚C for 10 min. Ice bath was followed for 5 min. The qPCR reaction system was: 2 μl reverse transcription product, 0.5 μl upstream primer, 0.5 μl downstream primer, 2X SYBR Green PCR Master Mix 10 μl, and sterilized and deionized water complemented to 20 μl. Reaction conditions were: 95˚C for 2 min, 95˚C for 50 sec, 60˚C for 45 sec, extension at 72˚C for 30 sec, and for a total of 40 cycles. The expression levels of TNF-α mRNA and TGF-β1 mRNA were detected with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal reference. The primer sequences are shown in Table I (the primers were synthesized and designed by Shanghai Gemma Company), and the experiment was repeated 3 times. The result was analyzed with $2^{\Delta\Delta-Cq}$ method (12).

Expression levels of TNF-α protein and TGF-β1 protein in myocardial tissues measured by western blot analysis. Cardiac muscle tissues (100 g) of mice were taken and RIPA was added lysate to extract the total protein. Then the extracted protein was boiled at 100˚C for 5 min to denaturate, then separated with 10% SDS-PAGE after being cooled, transferred to the PVDF membrane, and sealed with 5% of skim milk at room temperature for 1 h. Then the primary rabbit anti-mice polyclonal antibodies of TNF-α (1:500), TGF-β1 (1:500) and β-actin (1:1,000) (cat. nos. 17590-1-AP, 21898-1-AP, 14395-1-AP; ProteinTech Group, Inc.) were added at 4˚C for incubation overnight, then the secondary goat anti-rabbit polyclonal antibody (dil, 1:500; cat. no. SA00001-2; ProteinTech Group, Inc.) at room temperature for 1 h incubation, finally ECL developer was used to develop color.

Observation indicators. i) Eight-day survival rates of mice in each group were recorded and compared. ii) The pathological score in myocardial tissues of mice in each group was

Table II. Expression levels of TNF-α mRNA and TGF-β1 mRNA in myocardial tissues of mice in each group.

Factor	Blank group (n=20)	Control group (n=20)	VMC group (n=9)	MG-132 group (n=15)	F value	P-value
TNF-α	0.41±0.05[a,b]	0.41±0.05[a,b]	1.83±0.13	1.09±0.12[a]	779.6	<0.001
TGF-β1	0.51±0.06[a,b]	0.52±0.07[a,b]	1. 94±0.31	1.27±0.24[a]	204.0	<0.001

[a]P<0.05 compared to VMC group; [b]P<0.05 compared to MG-132 group. TGF-β1, transforming growth factor-β1; VMC, viral myocarditis.

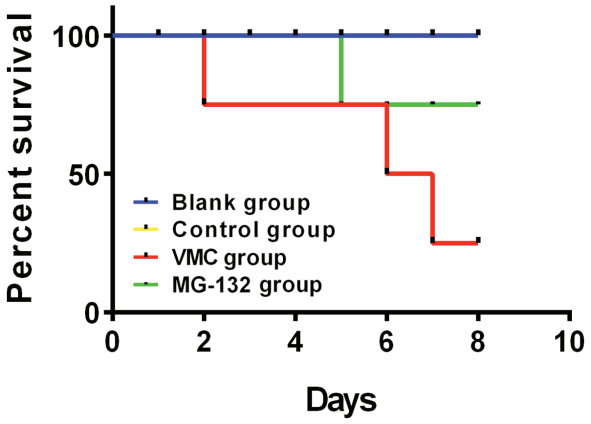

Figure 1. Comparison of survival rates of mice in each group. The survival rate of the blank group and the control group was 100%. The 8-day survival rates of the blank and control group were significantly higher than those of the VMC and MG-132 group, but the 8-day survival rate of MG-132 group was significantly higher than that of the VMC group, and the differences were statistically significant (P<0.05). VMC, viral myocarditis.

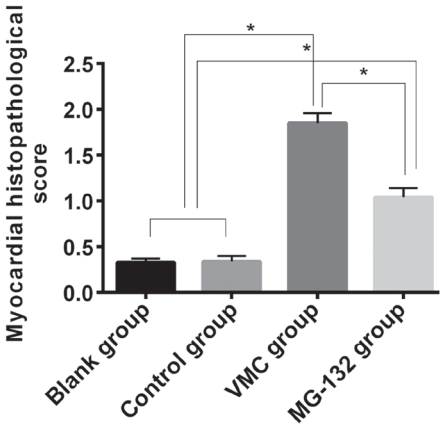

Figure 2. Comparison of myocardial histopathological scores of mice in each group. The myocardial histopathological scores of mice in the blank group and the control group were significantly lower than those in the VMC group and the MG-132 group, and the myocardial histopathological scores of mice in the MG-132 group were significantly lower than those in the VMC group, and the differences were statistically significant (*P<0.05). VMC, viral myocarditis.

evaluated and compared. iii) The expression levels of TNF-α mRNA and TGF-β1 mRNA in myocardial tissues of mice in each group were compared. iv) TNF-α protein and TGF-β1 protein in myocardial tissues of mice in each group were compared. v) Correlation analysis was performed between the pathological score and the protein expression levels of TNF-α and TGF-β1 in myocardial tissues of mice, and the protein expression levels of TNF-α and TGF-β1 in myocardial tissues.

Statistical analysis. SPSS 20.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis of the experimental data. The Chi-square test was used to compare the enumeration data. Kaplan-Meier curve was used for survival analysis. Measurement data were expressed as mean ± standard deviation. t-test was used for comparison between the two groups and univariate analysis of variance was used for multigroup comparison. LSD/t-test was used for postoperative comparison. Correlation was analyzed by Pearson's correlation analysis. GraphPad Prism 6 software (Hangzhou Aimeilv Biotechnology Co., Ltd.) was used to draw the experimental images. P<0.05 was considered to indicate a statistically significant difference.

Results

Comparison of survival rates of mice in each group. The survival rate of the blank group and the control group at day 8 was 100%. A total of 11 mice in the VMC group died at day 8, with a survival rate of 45%. A total of 5 mice in the MG-132 group died at day 8, with a survival rate of 75%. The 8-day survival rates of the blank group and control group were significantly higher than those of the VMC group and MG-132 group, but the 8-day survival rate of mice in the MG-132 group was significantly higher than that of the VMC group (P<0.05) (Fig. 1).

Histopathological scores of myocardial tissues of mice in each group. There was no inflammatory cell infiltration in myocardial tissues of mice in blank or control group. There was a large amount of inflammatory cell infiltration in myocardial tissues of mice in VMC group. The inflammatory cells in the myocardial tissues of mice in MG-132 group were significantly reduced compared with those in VMC group. The myocardial histopathological scores of mice in blank and the control group were respectively 0.33±0.04 and 0.34±0.06, the myocardial histopathological score of mice in the VMC group was 1.85±0.11, and was 1.04±0.10 in the MG-132 group. The myocardial histopathological scores of mice in the blank and the control group were significantly lower than those in VMC and MG-132 group, and myocardial histopathological score of mice in MG-132 was significantly lower than that in VMC group (P<0.05) (Fig. 2).

Expression levels of mRNA of TNF-α and TGF-β1 in myocardial tissues of mice in each group. There was no significant

Table III. Expression levels of TNF-α protein and TGF-β1 protein in myocardial tissues of mice in each group.

Factor	Blank group (n=20)	Control group (n=20)	VMC group (n=9)	MG-132 group (n=15)	F value	P-value
TNF-α (ng/l)	1.15±0.59[a,b]	1.14±0.62[a,b]	2.87±0.65	1.84±0.58[a]	21.36	<0.001
TGF-β1(ng/ml)	1.05±0.41[a,b]	1. 03±0.42[a,b]	2.14±0.61	1.57±0.59[a]	14.07	<0.001

[a]P<0.05 compared to VMC group; [b]P<0.05 compared to MG-132 group. TGF-β1, transforming growth factor-β1; VMC, viral myocarditis.

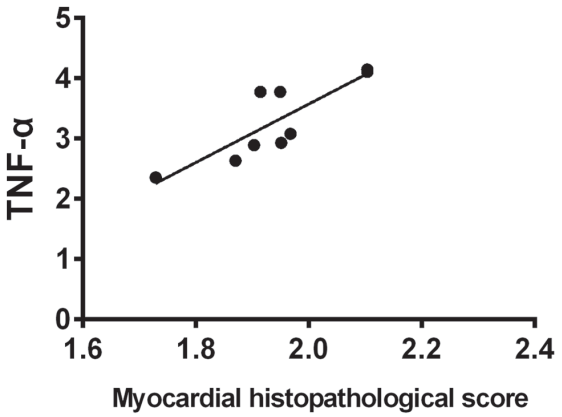

Figure 3. Correlation analysis of expression of TNF-α protein in myocardial tissues and pathological scores of mice in myocardial tissues. Pearson's correlation analysis showed that there was a positive correlation between the pathological score of mice in myocardial tissues and the expression of TNF-α protein in myocardial tissues (r=0.843, P<0.05). TNF-α, tumor necrosis factor-α.

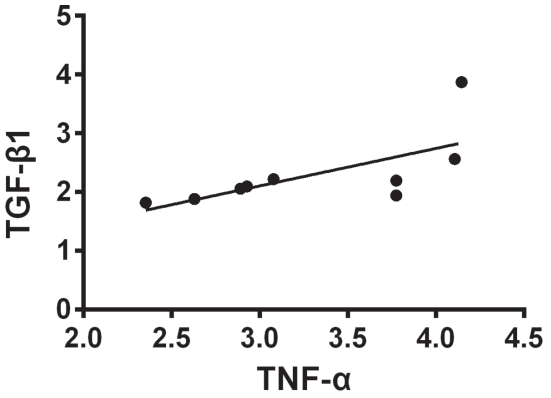

Figure 5. Correlation analysis of protein expression between TNF-α and TGF-β1 in myocardial tissues of VMC mice. Pearson's correlation analysis showed a positive correlation between the expression levels of TNF-α protein and TGF-β1 protein in myocardial tissues of VMC mice (r=0.672, P<0.05). TNF-α, tumor necrosis factor-α; TGF-β1, transforming growth factor-β1; VMC, viral myocarditis.

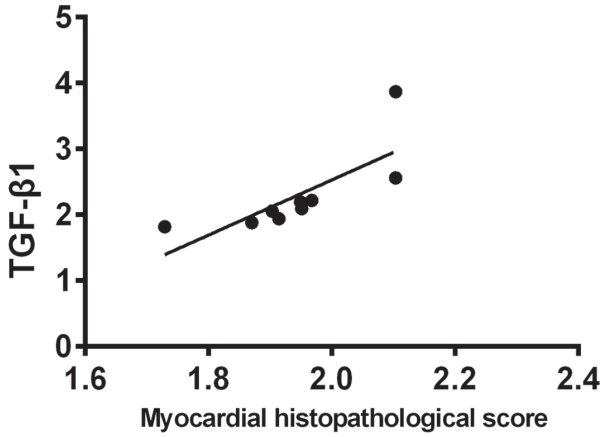

Figure 4. Correlation analysis between the pathological score of mice and expression of TGF-β1 protein in myocardial tissues. Pearson's correlation analysis indicated that the pathological score in myocardial tissues of mice was positively correlated with the expression of TGF-β1 protein in myocardial tissues (r=0.763, P<0.05). TGF-β1, transforming growth factor-β1.

difference in expression levels of TNF-α mRNA and TGF-β1 mRNA between the blank group and the control group (P>0.05), but were significantly lower than those in the VMC group and the MG-132 group. However, expression levels of TNF-α mRNA and TGF-β1 mRNA in myocardial tissues of MG-132 group were significantly lower than those of the VMC group (P<0.05) (Table II).

Expression levels of TNF-α protein and TGF-β1 protein in myocardial tissues of mice in each group. There was no significant difference in the expression levels of TNF-α protein and TGF-β1 protein between blank and control group (P>0.05), but both were significantly lower than those of VMC group and the MG-132 group. However, expression levels of TNF-α and TGF-β1 protein in myocardial tissues of mice in the MG-132 group were significantly lower than those in the VMC group (P<0.05) (Table III).

Correlation analysis of the pathological score in myocardial tissues and expression levels of TNF-α protein and TGF-β1 protein in myocardial tissues of mice and the protein expression levels between TNF-α and TGF-β1 in myocardial tissues of VMC mice. The expression levels of TNF-α protein and TGF-β1 protein in myocardial tissues were positively correlated with the pathological score of myocardial tissues (r=0.843, P<0.05; r=0.763, P<0.05), and there was a positive correlation between expression levels of TNF-α protein and TGF-β1 protein of VMC mice in myocardial tissues (r=0.672, P<0.05) (Figs. 3-5).

Discussion

As a viral infectious disease, VMC currently has no specific effective treatment in clinical practice, thus, VMC is one of the most challenging diseases in the diagnosis and treatment of cardiovascular field at present (13). Besides, there are also

great controversies regarding the pathogenesis of VMC, most of which support cytokine theory and immune theory (14). As an important protein quality control system in eukaryotic cells, UPS has been found to play a very important role in various viral infectious diseases in recent years. Specifically, through UPS, viruses can replicate and lead to oxidative stress in host cells and eventually cause cell damage (15-17). For example, studies (18) have found that the normal UPS pathway is involved in the replication of vaccinia virus. However, there are few studies on UPS in VMC, and no detailed description of its mechanism has been made.

In our study, the effects of UPS inhibitor MG-132 intervention on myocardial cells, TNF-α and TGF-β1 in VMC mice were investigated. The results showed that the mortality of the VMC group was significantly higher than that of the blank group, control group and MG-132 group ($P<0.05$), suggesting VMC mice had higher mortality rates, but the mortality of VMC mice can be significantly reduced after the intervention of UPS inhibitor MG-132. In addition, the expression levels of myocardial histopathological scores, mRNA and protein of TNF-α and TGF-β1 in the blank group and the control group were significantly lower than those in the VMC group and the MG-132 group, and the myocardial histopathological scores, mRNA and protein of TNF-α and TGF-β1 in the MG-132 group were significantly lower than those in the VMC group ($P<0.05$). This suggests that the intervention of UPS inhibitor MG-132 can effectively improve the inflammatory infiltration in myocardial tissues of VMC mice and reduce the expression levels of inflammatory factors in myocardial tissues. UPS plays an important role in inflammatory response. For example, a previous study (19) found that UPS was involved in the response injury of hepatitis B coronavirus to a certain extent and could effectively alleviate the occurrence of inflammatory response.

Other studies (20) have shown that UPS inhibitor MG-132 can inhibit the AKT and ERK pathways, thereby alleviating the inflammatory response and inhibiting the progression of heart failure. Although these studies did not directly confirm our conclusions, they can also show that the intervention of UPS inhibitor MG-132 does have a certain alleviating effect on the inflammatory response. Therefore, previous studies (21) suggested that inhibition of the process of heart failure by UPS inhibitors may be achieved by reducing oxidative stress. However, some studies (22) have obtained different results when different UPS inhibitors were applied in the intervention of ischemic cardiomyopathy, and the reason is not clear at present. We speculate that it may be because UPS inhibitors are involved in different molecular reactions, so they have different effects on inflammatory reactions or oxidative stress reactions. Finally, we analyzed the correlation between the pathological score in myocardial tissues of mice and the protein expression levels of TNF-α and TGF-β1 in myocardial tissues, as well as the protein expression levels between TNF-α and TGF-β1 in myocardial tissues of VMC mice. The results showed that the expression levels of TNF-α and TGF-β1 proteins in myocardial tissues were positively correlated with the pathological score in myocardial tissues of mice, and the expression levels of TNF-α protein and TGF-β1 protein in myocardial tissues of VMC mice were also positively correlated. Previous studies (23) have shown that the regulation of TGF-β can effectively stimulate the release of TNF-α in human monocytes. Although it is not a study of the myocardial tissues, it also confirms our conclusion.

In recent years, the role of UPS in cardiovascular diseases has been gradually recognized and discovered (24). After the establishment of VMC mouse model, we also found that the UPS inhibitor MG-132 can significantly alleviate the myocardial injury of VMC mice, reduce the expression levels of inflammatory factors in myocardial tissues, and improve the survival rate of mice. UPS inhibitor MG-132 may be a new treatment scheme for VMC. However, our study also has some shortcomings. We did not further explore the mechanism of action between TNF-α and TGF-β1, or describe in detail how MG-132 reduced the inflammatory response in VMC mice. This will be further explored in our subsequent experiments.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

HZ, JY and HS performed RT-qPCR. YZ and JW were responsible for western blot analysis. JZ and BM contributed to analysis of the observation indexes. HZ wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Central Hospital of Zibo (Zibo, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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