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Research article

Dexmedetomidine suppressed the biological behavior of RAW264.7 cells treated with LPS by down-regulating HOTAIR

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ABSTRACT

Background: Previous studies have revealed dexmedetomidine have potential protective effects on vital organs by inhibiting the release of inflammatory cytokines. To investigate the effects of dexmedetomidine on sepsis, especially in the initial inflammatory stage of sepsis. RAW264.7 cells were used as the cell model in this study to elucidate the underlying mechanisms.
Methods: In this study, we conducted several assays to investigate the mechanisms of dexmedetomidine and HOTAIR in sepsis. Cell viability was assessed using the CCK-8 kit, while inflammation responses were measured using ELISA for IL-1β, IL-6, and TNF-α. Additionally, we employed qPCR, MeRIP, and RIP to further explore the underlying mechanisms.
Results: Our findings indicate that dexmedetomidine treatment enhanced cell viability and reduced the production of inflammatory cytokines in LPS-treated RAW264.7 cells. Furthermore, we observed that the expression of HOTAIR was increased in LPS-treated RAW264.7 cells, which was then decreased upon dexmedetomidine pre-treatment. Further investigation demonstrated that HOTAIR could counteract the beneficial effects of dexmedetomidine on cell viability and cytokine production. Interestingly, we discovered that YTHDF1 targeted HOTAIR and was upregulated in LPS-treated RAW264.7 cells, but reduced in dexmedetomidine treatment. We also found that YTHDF1 increased HOTAIR and HOTAIR m6A levels.
Conclusions: Collectively, our results suggest that dexmedetomidine downregulates HOTAIR and YTHDF1 expression, which in turn inhibits the biological behavior of LPS-treated RAW264.7 cells. This finding has potential implications for the prevention and treatment of sepsis-induced kidney injury.

1. Introduction

Sepsis is a syndrome of physiological, pathological, and biochemical abnormalities caused by a series of physiological responses to infection, leading to organ dysfunction [1,2]. In the initial phase of sepsis, the body's inflammatory response is activated, and many pro-inflammatory cytokines are released [3–5], such as tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6),

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and late inflammatory mediators, such as high mobility group box 1 and prostaglandins, which cause the body's immune defense system to be overactivated. At the same time, immune cells such as T lymphocytes also release large amounts of cytokines, accompanied by oxidative stress imbalance [6]. These mechanisms collectively activate endothelial cells, leading to increased release of cytokines, nitric oxide, platelet activating factors, coagulation factors, and other factors, which further aggravates sepsis, leading to septic shock and ultimately death.

To date, more than 100 types of post-transcriptional modifications have been identified in RNAs [7,8], and N6-methyladenosine nucleotide (m6A) RNA methylation is one of the most common modifications, accounting for more than 50% of total methylated nucleotides and 0.1%–0.4% of all adenosines in cellular RNA [9]. RNA binding proteins that recognize m6A sites and determine the fate of m6A-modified RNAs are called m6A "readers" [10]. According to their mode of action, they can be divided into direct and indirect acting proteins. Direct acting proteins, which include proteins with the YT521-B homology (YTH) domain, such as YTHDF1-3, YTHDC1 and YTHDC2, are the most important functional proteins [11]. They have an affinity for m6A-containing mRNA that is 10–50 times higher than that of non-methylated mRNA. When cells are stimulated under different conditions, different members of the YTH domain family proteins interact with m6A sites to exert different effects on mRNA gene expression. YTH N6-methyladenosine RNA binding protein 1 (YTHDF1), which contains the YTH domain, can bind to m6A sites near the stop codon and then interact with the translation initiation factor eIF3 to stimulate mammalian translation, ultimately promoting translation [12,13]. (Among them, YTH domain family 1 (YTHDF1) can promote protein synthesis by interacting with the translation mechanism. The translation promotion mediated by YTHDF1 increases translation efficiency, ensuring the production of effective protein from dynamically transcribed m6A-labeled transcripts.)Long non-coding RNAs (lncRNAs) are a kind of RNA without protein-encoding function, which is about 200 nt in length and involved in various life activities [14]. It is also closely related to the occurrence and development of many diseases. LncRNA HOX transcript antisense RNA (HOTAIR) was the first RNA found to possess the *trans*-regulatory effect [15].

The pathogenesis and development mechanism of sepsis are complex. In recent years, many scholars have conducted extensive research on the pathogenesis of sepsis as a therapeutic target, but the incidence and mortality rate of sepsis have not been effectively improved. This study focuses on HOTAIR as a starting point to explore its biological function in the progression of sepsis, with the aim of providing scientific basis for the treatment of sepsis.

2. Materials and methods

2.1. Cell culture and transfection

The murine stable cell line RAW264.7 (available at ATCC) was grown in Dulbecco' s Modified Eagle's medium (DMEM) (Thermo Scientific, USA) supplemented with 10 % fetal bovine serum (FBS) and 1% Penicillin-Streptomycin solution (Thermo Scientific, USA) at 37 °C with 5% CO_2 [16,17]. HOTAIR and YTHDF1 was amplified from RAW264.7 cells and cloned into pLVX-IRES-Neo vector and pcDNA3 vector, respectively (Invitrogen, USA). While, the siHOTAIR, siYTHDF1 and their respective negative control were obtained from (Thermo Scientific, USA). All transfection were before LPS induction. Briefly, the experimental group was pretreated with 1 μM dexmedetomidine (Hengrui, China) for 2 h, then HOTAIR or TYTHDF1 transfection by using Lipofectamine 2000 (Invitrogen, USA) reagent for indicate time, LPS (100 ng/mL) induction were treated after 12 h of dexmedetomidine pretreatment in RAW264.7 cells.

2.2. Cell viability

The RAW264.7 cells under different treatment were cultured in 24-well plates at a density of 3×10^5 cells per well. CCK-8 solution (Beyotime, China) was added to the cell medium and incubated for 4 h at 37 °C [18]. The absorbance values were then measured at 450 nm using the Thermo Scientific Microplate Reader (BioTek, USA).

2.3. Determination of IL-1β, IL-6, and TNF-α

IL-1β, IL-6, and TNF-α levels in culture media of the cells were determined using IL-1β, IL-6, and TNF-α ELISA kits (Thermo Scientific, USA) according to the manufacturer's instructions [19]. The absorbance values were then measured at 450 nm using the Thermo Scientific Microplate Reader (BioTek, USA). Levels of tumor necrosis factor-α (TNF-α) were measured in duplicate in the murine stable cell line RAW264.7 using a commercial ELISA kit (Thermo Scientific, USA, intra-assay coefficient of variation [CV] < 10%, inter-assay CV<15%, detection sensitivity 1.0 pg/mL). Levels of interleukin (IL)-6 were measured in duplicate in the murine stable cell line RAW264.7 using the ELISA method (Thermo Scientific, USA, intra-assay CV <10%, inter-assay CV <15%, detection sensitivity <0.1 pg/mL). Levels of interleukin (IL)-1βwere measured in duplicate in the murine stable cell line RAW264.7 using the ELISA method (Thermo Scientific, USA, intra-assay CV <10%, inter-assay CV <15%, detection sensitivity 0.1 pg/mL).

2.4. Western blot

The RAW264.7 cells were washed with phosphate-buffered saline (PBS) and then lysed with RIPA lysis buffer (Beyotime, China) and sonicated, then denatured by incubation for 5 min at 95 °C in a sample buffer (2% SDS, 10% glycerol, 60 mM Tris (pH 6.8), 5% β-mercaptoethanol, and 0.01% bromophenol blue). After that, the samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to the nitrocellulose transfer membrane (PALL China, Shanghai) using the PyxisTM Gel Processor. After being blocked in 0.05% Tween-PBS with 5% skim milk for 30 min, the membranes were subjected to immunoblot

with the primary antibodies and subsequently with HRP-conjugated secondary antibodies. After washing with PBST (PBS buffer containing 0.05% Tween 20) for 30 min, the membranes were then developed with ECL (Thermo) and imaged by the Tannon5200 system (Tannon Inc., Shanghai, China). Antibodies were used, including rabbit anti-YTHDF1 monoclonal antibody (1:1000 , Cell signaling technology), mouse anti-GAPDH monoclonal antibody (1:1000,Abcam). Secondary antibodies included HRP-conjugated goat anti-rabbit IgG antibody and anti-mouse IgG antibody (1:2000, Southern Biotech, USA).

2.5. Methylated RNA immunoprecipitation assay (MeRIP)

To determine the m6A modifications of HOTAIR, we performed the Methylated RNA immunoprecipitation (MeRIP) assay with a Magna MeRIPTM m6A kit (Sigma, USA) according to the manufacturer's instructions. The collected solutions were used to confirm the m6A level via qRT-PCR assay. The primers used in this study are presented in Table 1.

2.6. RNA immunoprecipitation assay (RIP)

RAW264.7 cells were collected and subsequently lysed with RIP lysis buffer. The beads (Thermo Scientific, USA) were combined with rabbit anti-YTHDF1 antibody (Cell signaling technology) and anti-rabbit IgG antibody (Cell Signaling Technology), and washed twice with RIP wash buffer. Lysis buffer and beads were incubated overnight with the lysed cells at 4 °C. The following day, the beads were collected and washed with RIP wash buffer, and the RNA complexes were isolated through phenol-chloroform extraction and analyzed via qRT-PCR. The primers used in this study are presented in Table 1 , which were designed using Primer6.

2.7. Real-time quantitative PCR analysis

Following the manufacturer's protocol, RNA was extracted from RAW264.7 cells using Trizol reagent (Invitrogen), and RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). cDNA was then synthesized using a reverse transcription kit (Invitrogen, USA) according to the manufacturer's instructions. For cDNA synthesis, the reaction mixtures were incubated at 16 °C for 30 min, at 42 °C for 30 min and at 85 °C for 5 min and then held at 4 °C. The quantitative detection of HOTAIR was performed using the TaqMan® Gene Expression Assay and the reaction mixtures were incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The primers of HOTAIR were listed in Table 1. The $2^{-\Delta\Delta}$ Ct method was employed to calculate relative gene expression, and GAPDH was used as an endogenous control.

2.8. Statistical analysis

All the data were graphed, and statistical analyses were performed using Prism 8 software (GraphPad, La Jolla, CA, USA). Comparisons between two groups' means were performed with a two-tailed Student's t-test, whereas multiple comparisons were conducted by one-way analysis of variance (ANOVA). The differences were considered statistically significant at p values of <0.05 or <0.01.

3. Results

1. Dexmedetomidine promoted LPS induced cell proliferation and cell viability in RAW264.7 Cells

As indicated in our findings, the cell viability of RAW264.7 cells remained unaffected during the initial 24-h period of 100 ng/mL LPS treatment (Fig. 1A). However, we observed toxicity between 48 and 96 h following treatment, resulting in a noteworthy decline in both cell viability (Fig. 1A) and proliferation (Fig. 1B). To investigate the effect of dexmedetomidine on LPS-treated RAW264.7 cells, we pretreated the cells with 1 μM dexmedetomidine for 12 h, followed by stimulation with LPS (100 ng/mL) for 24, 48, 72, and 96 h, respectively. Our results demonstrated a substantial enhancement in cell proliferation and viability after 72–96 h of dexmedetomidine treatment (Fig. 1A and B).

2. Dexmedetomidine Reduced the Expression of HOTAIR in LPS-Treated RAW264.7 Cells

To investigate the regulatory effects of dexmedetomidine on LPS-induced cell proliferation and cell viability, we conducted an

Table 1
The primers of qRT-PCR used in this study.

Primers	Forward (5′-3′)	Reverse (5′-3′)
qPCR primers		
HOTAIR	TCCAGATGGAAGGAACTCCAGACA	ATAGATGTGCGTGGTCAGATCGCT
YTHDF1	GCATCAGAAGGATGCAGTTCATG	GATGGTGGATAGTAACTGGACAG
GAPDH	ACTCCACTCACGGCAAATTC	TCTCCATGGTGGTGAAGACA

The primers was designed using Primer6.

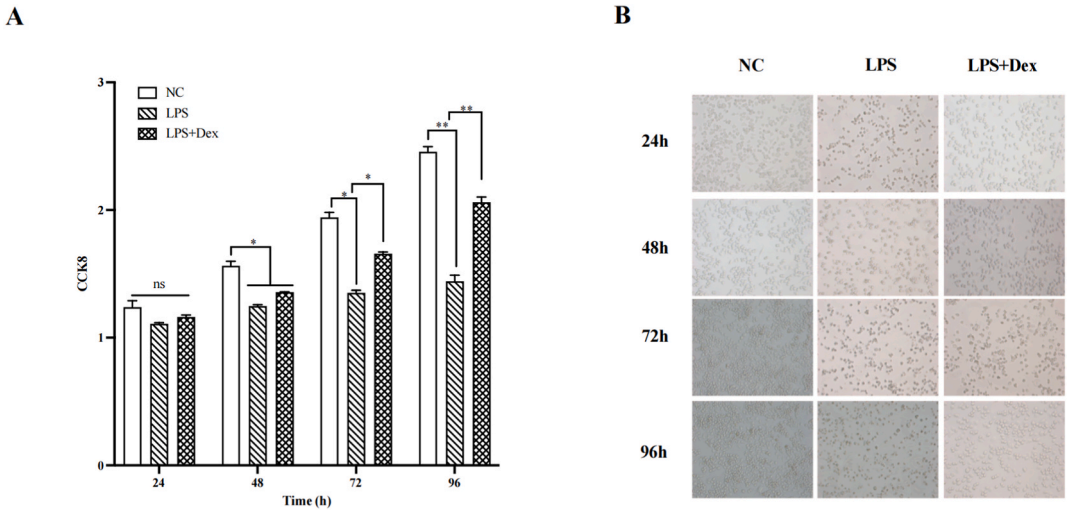

Fig. 1. Dexmedetomidine promoted LPS induced cell proliferation and cell viability in RAW264.7 cells.
RAW264.7 cells were pretreated with 1 μM dexmedetomidine for 12 h and then followed by stimulation with LPS (100 ng/mL) for 24, 48, 72, and 96 h, respectively. (A) The cell viability of RAW264.7 cells were measured by CCK-8 assay. (B) The cell images were observed under a microscope (B).

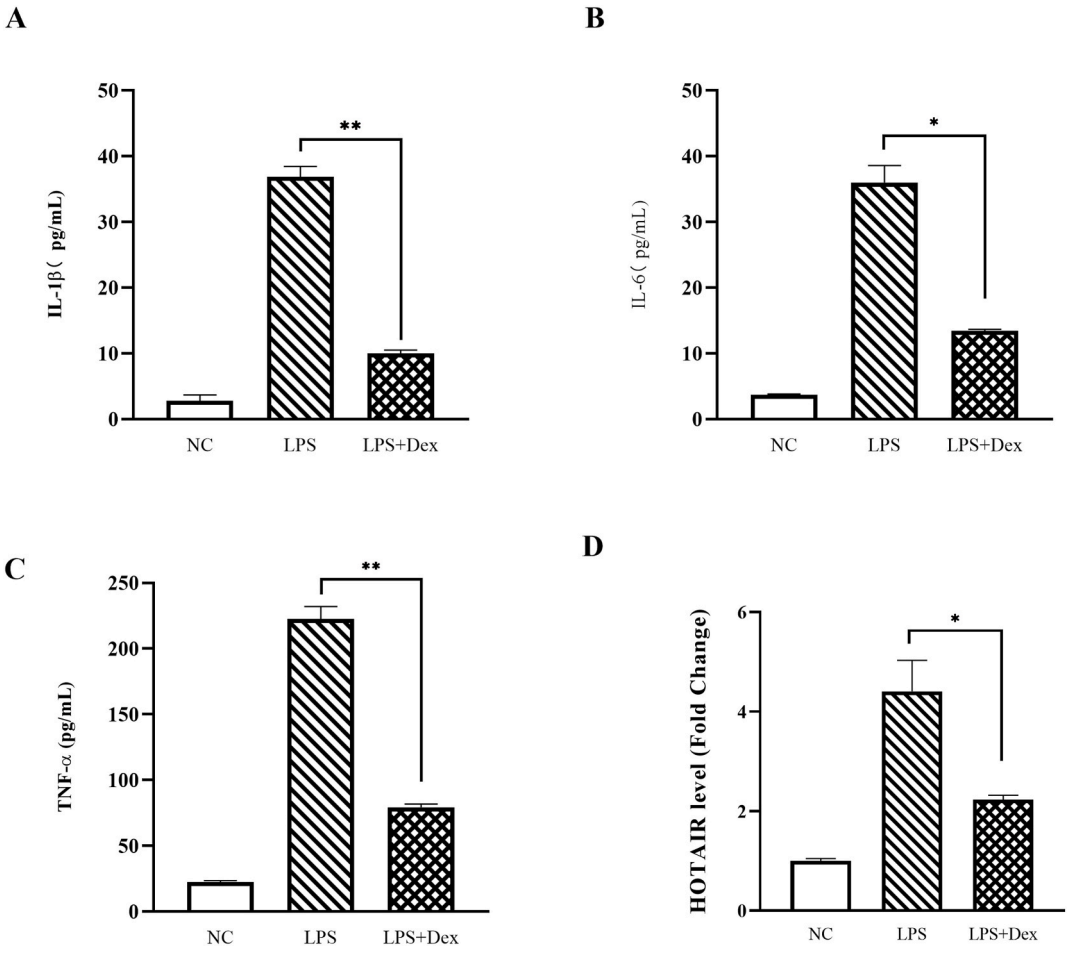

Fig. 2. Dexmedetomidine Reduced the Expression of HOTAIR in LPS-Treated RAW264.7 Cells.
The expression of IL-1β (A), IL-6 (B), and TNF-α (C) were measured using ELISA. (D) The expression of HOTAIR was detect by qRT-PCR.

experiment where RAW264.7 cells were pretreated with dexmedetomidine (1 μM) for 24 h prior to treatment with 100 ng/mL LPS. We first evaluated the inflammatory response by measuring the expression of cytokines (IL-1β, IL-6, and TNF-α) using ELISA. As shown in Fig. 2A–C, the production of cytokines in RAW264.7 cells treated with LPS was significantly reduced by dexmedetomidine. Specifically, dexmedetomidine pretreatment resulted in a 2.71-fold and 1.92-fold downregulation of IL-1β and TNF-α levels ($p < 0.01$). We also observed that the upregulation of HOTAIR expression in LPS-treated RAW264.7 cells was reversed by dexmedetomidine (Fig. 2D).

3. HOTAIR abolish dexmedetomidine in improving cell viability and inhibition of cytokines production

Based on the aforementioned research findings, we hypothesize that dexmedetomidine's ability to improve sepsis may be linked to its regulation of HOTAIR. To test this hypothesis, we overexpressed or knocked down HOTAIR in LPS-treated RAW264.7 cells. Our results, presented in Fig. 3, demonstrate that knocking down HOTAIR significantly suppressed the induction of cytokines (IL-1β, IL-6, and TNF-α) in LPS-treated RAW264.7 cells ($p < 0.01$), achieving a similar effect to dexmedetomidine pretreatment (Fig. 2A–C). Furthermore, we transfected pHOTAIR or siHOTAIR into RAW264.7 cells treated with LPS (100 ng/mL, 48 h stimulation) under dexmedetomidine pretreatment and found that increasing HOTAIR expression reversed the beneficial effects of dexmedetomidine on cell activity ($p < 0.01$) (Fig. 4A and B). However, transfection with siHOTAIR slightly enhanced cell ability ($p < 0.05$) (Fig. 4B). Our findings suggest that increasing HOTAIR levels negates the inhibitory effect of dexmedetomidine on cytokine production (IL-1β, IL-6, and TNF-α) in RAW264.7 cells treated with LPS (Fig. 4C–E).

4. Dexmedetomidine up-regulated the m6A level of HOTAIR in LPS-treated RAW264.7 cells.

Further, we investigated the m6A level of HOTAIR in RAW264.7 cells with different interventions. As shown, HOTAIR m6A

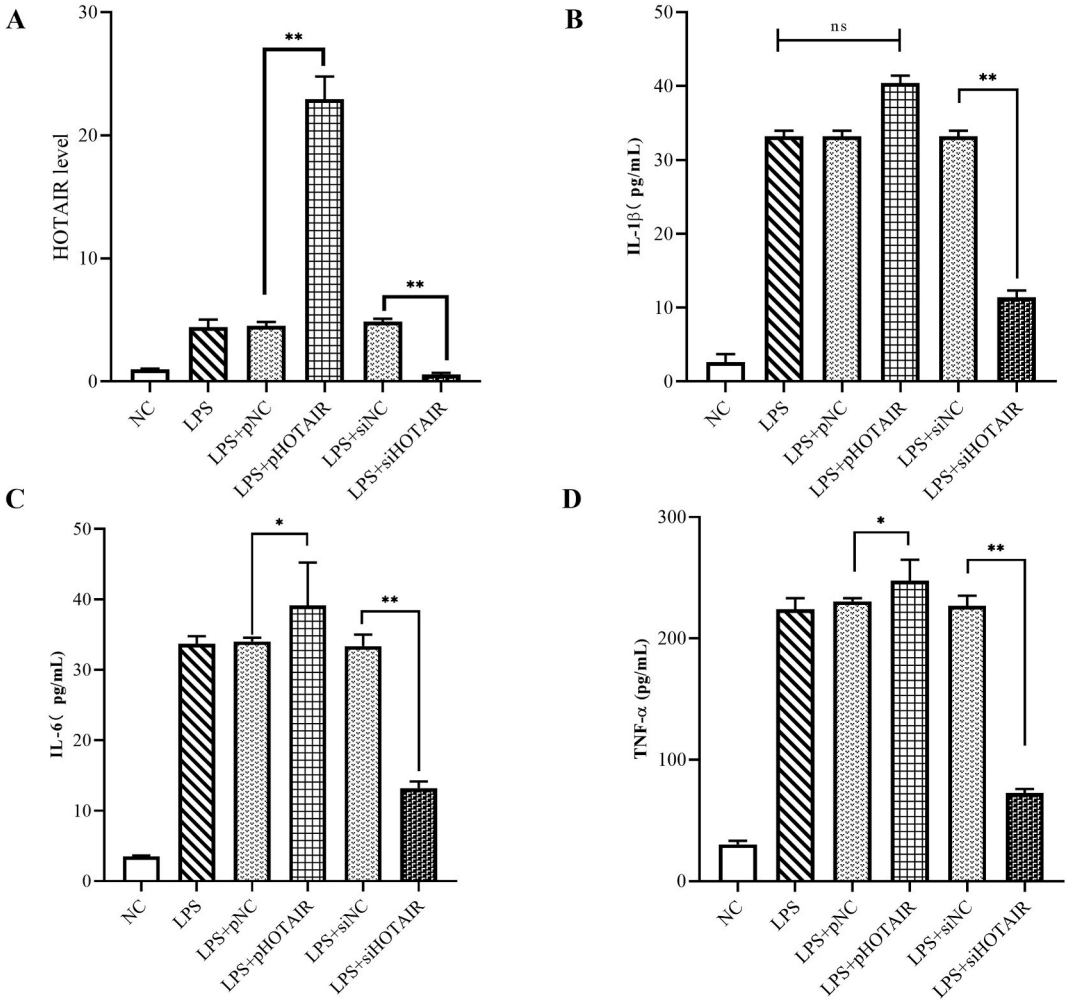

Fig. 3. HOTAIR knock down significantly suppressed IL-1β, IL-6, and TNF-α induction in LPS treated RAW264.7 cells. (A) The expression of HOTAIR was detect by qRT-PCR. The expression of IL-1β (B), IL-6 (C), and TNF-α (D) were measured by using ELISA.

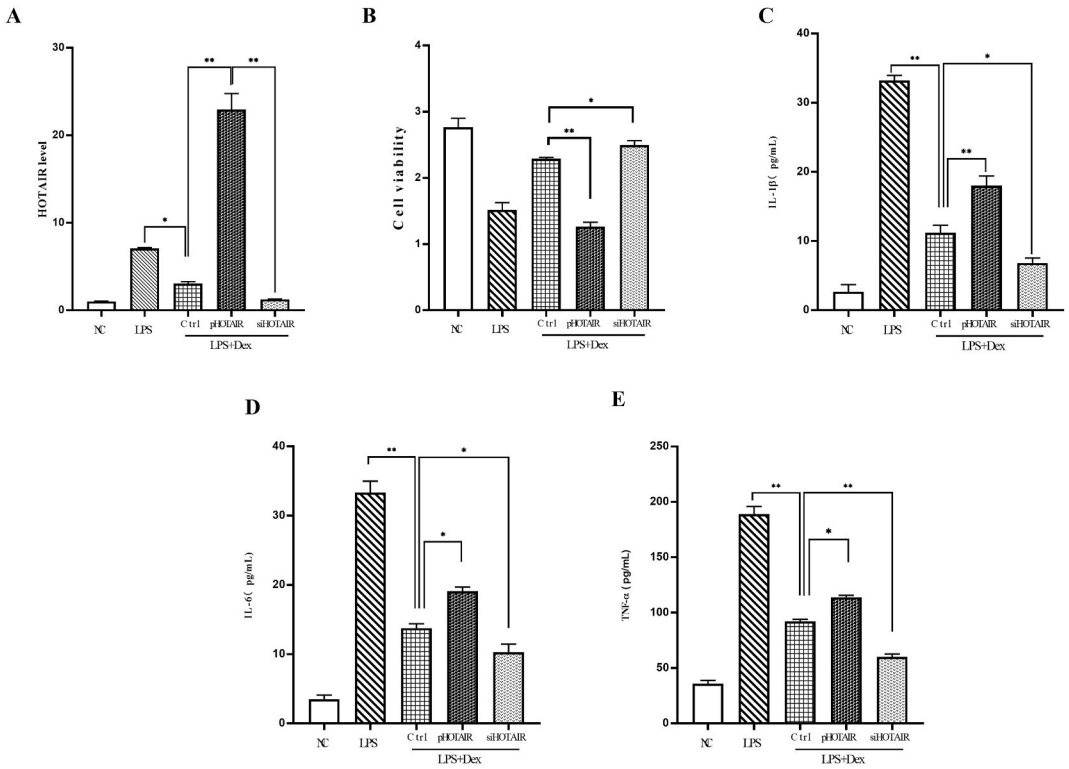

Fig. 4. HOTAIR abolish dexmedetomidine in improving cell viability and inhibition of cytokines production. (A) The expression of HOTAIR m6A level was detect by MeRIP assay via qRT-PCR. (B) The cell viability of RAW264.7 cells were measured by CCK-8 assay. The expression of IL-1β (C), IL-6 (D), and TNF-α (E) were measured using ELISA.

enrichment was increased in RAW264.7 cells under LPS stimulation, whereas dexmedetomidine pretreatment downregulated the m6A level of HOTAIR (Fig. 5A). YTHDF1, act as m6A readers by directly binding to the m6A modification sites on RNAs and facilitating their translation and degradation. To investigate whether HOTAIR associates with YTHDF1, we performed RIP-qPCR experiments and found that HOTAIR was significantly enriched by anti-YTHDF1 compared with anti-IgG (Fig. 5B), indicating that HOTAIR could directly target YTHDF1. In addition, we measured the level of YTHDF1 by qPCR and WB and found that YTHDF1 was increased in LPS-treated RAW264.7 cells, while dexmedetomidine pretreatment downregulated the level of YTHDF1 in LPS-treated RAW264.7 cells (Fig. 5C). Furthermore, we performed qPCR and MeRIP-qPCR to determine the HOTAIR and HOTAIR m6A levels and found that inhibiting YTHDF1 resulted in a downregulation of the HOTAIR and HOTAIR m6A levels, while elevating YTHDF1 increased the HOTAIR and HOTAIR m6A levels (Fig. 5D–F). Our data suggests that YTHDF1 may increase the transcription level of HOTAIR by enhancing the m6A modification in HOTAIR.

4. Discussion

Sepsis is a syndrome of pathophysiological and biochemical abnormalities caused by infection, leading to organ dysfunction [20,21]. In the early stage of sepsis, the body's inflammatory response is activated and many pro-inflammatory cytokines are released, such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), resulting in overactivation of the immune system [22,23]. At the same time, immune cells such as T lymphocytes also release large amounts of cytokines, accompanied by imbalance of oxidative stress. These mechanisms collectively activate endothelial cells, further aggravating sepsis and ultimately leading to septic shock and death.

In recent years, numerous studies conducted by scholars both domestically and internationally have demonstrated the protective effects of dexmedetomidine on several vital organs [24,25]. These effects are thought to be attributed to its anti-inflammatory and anti-oxidative properties, as well as its ability to inhibit apoptosis. In this study, we found that the viability of RAW264.7 cells decreased after intervention with LPS, especially at 48 h after LPS treatment, the cell viability significantly decreased compared to the control group (Fig. 1A, $p < 0.05$). Additionally, Basso F G et al. also found that LPS intervention can significantly affect cell viability and proliferation [26]. Furthermore, we discovered that dexmedetomidine could promote cell proliferation, viability and inhibition of cytokines (IL-1β, IL-6, and TNF-α) in LPS-treated RAW264.7 cells (Figs. 1 and 2A–C). In LPS-treated RAW264.7 cells, HOTAIR expression was upregulated (Fig. 2D), which could be reversed by dexmedetomidine. Further experiments have confirmed that inhibiting HOTAIR can mimic the effects of dexmedetomidine in promoting cell proliferation and reducing inflammatory cytokine

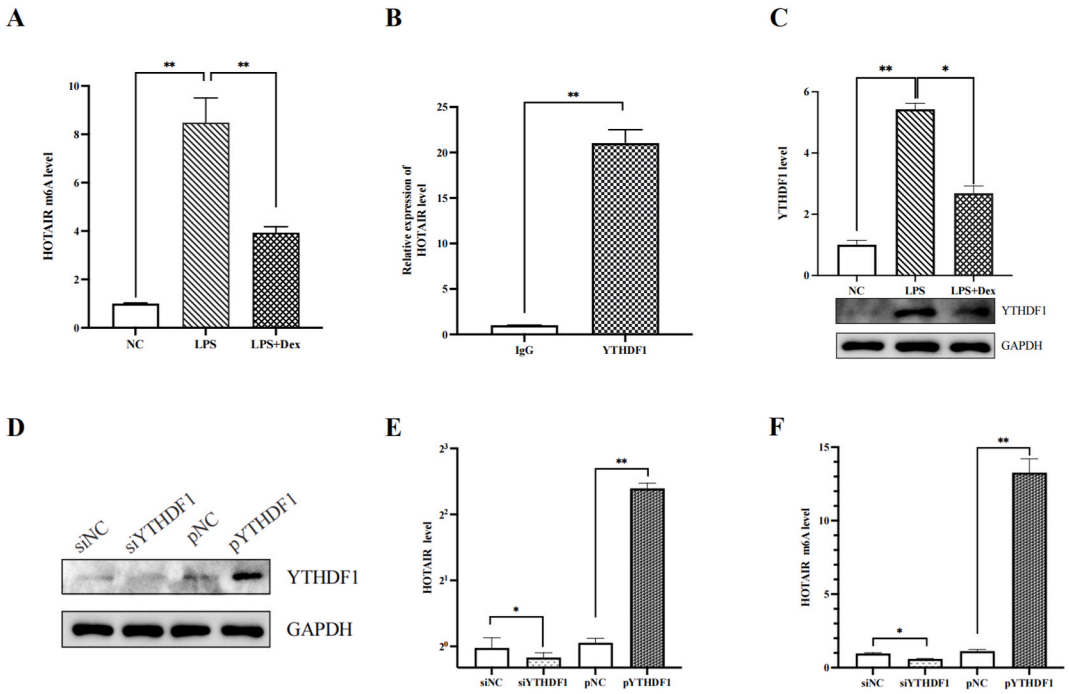

Fig. 5. Dexmedetomidine up-regulated the m6A level of HOTAIR in LPS-treated RAW264.7 cells. (A) The expression of HOTAIR m6A level was detect by MeRIP-qPCR. (B) HOTAIR was significantly enriched by YTHDF1 compared with IgG. (C) The expression of YTHDF1 was detect by qRT-PCR and WB in LPS-treated RAW264.7 cells. RAW264.7 cells were transfected with siYTHDF1 (300 nM) or pYTHDF1 (10 μg) in a 100-mm dish, and the cell lysate was divided into three parts: one for WB to determine the expression of YTHDF1 (D), and the other two for qRT-PCR to assess HOTAIR expression (E) and MeRIP-qPCR to determine the m6A level on HOTAIR (F).

production (Fig. 3). Therefore, our data suggests that HOTAIR may play an important role in the ability of dexmedetomidine to alleviate LPS-induced injury. In our experiments with RAW264.7 cells treated with LPS and pre-treated with dexmedetomidine, we demonstrated through modulation of HOTAIR levels that increasing HOTAIR levels significantly inhibited cell viability, while decreasing HOTAIR levels significantly improved cell viability (as shown in Fig. 4B). Additionally, our results demonstrate that the improvement of LPS-induced inflammation by dexmedetomidine can be reversed by increasing HOTAIR levels (Fig. 4C–E).

To explore the mechanism by which dexmedetomidine improves sepsis, we focused on HOTAIR and found that HOTAIR m6A enrichment was decreased in RAW264.7 cells under LPS stimulation. However, dexmedetomidine intervention increased the m6A level of HOTAIR in LPS-treated RAW264.7 cells (Fig. 5A). The study also found that dexmedetomidine negatively regulated the enrichment of m6A modification of HOTAIR in LPS-treated RAW264.7 cells. YTHDF1, an m6A reader, which are characterized by the presence of a conserved YTH domain that binds to methylated RNA. Further, our results demonstrate that YTHDF1 was directed target to HOTAIR (Fig. 5B). Meanwhile, the level of YTHDF1 was demonstrated increased in LPS treated RAW264.7 cells and reduced in dexmedetomidine pretreatment (Fig. 5C). In addition, YTHDF1 increase the HOTAIR and HOTAIR m6A levels (Fig. 5D–F). Taken together, our data suggests that YTHDF1 increase the transcription level of HOTAIR might due to its ability to enhancing the m6A modification in HOTAIR.

In summary, this study reveals a new mechanism for dexmedetomidine against LPS-induced injury, in which dexmedetomidine downregulates YTHDF1, reduce m6A modification of HOTAIR, and inhibits the expression of HOTAIR. This finding provides a theoretical basis for exploring new therapies for sepsis. The results of this study help further understand the mechanism of dexmedetomidine protecting against sepsis and provide potential new targets for the clinical treatment of sepsis. However, it is important to note that while our study has revealed the involvement of dexmedetomidine in regulating the progression of sepsis by modulating HOTAIR and YTHDF1 in RAW264.7 cells, it has not yet been validated in animal models. Validating these findings in animal models should be a key focus for future investigations.

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Data availability statement

The data used and analyzed in the current study are available from the corresponding author upon a reasonable request.

Ethics statement

None.

CRediT authorship contribution statement

Qin Liu: Writing – original draft. **Guang-Hu Yang:** Writing – original draft, Formal analysis. **Nai-Zhi Wang:** Supervision, Methodology, Formal analysis. **Xin-Cheng Wang:** Writing – original draft, Formal analysis, Conceptualization. **Zhao-Long Zhang:** Writing – original draft, Supervision, Methodology, Formal analysis. **Lu-Jun Qiao:** Writing – review & editing. **Wen-Juan Cui:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27690.

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