Inflammation, Vol. 42, No. 2, April 2019 (© 2018)
DOI: 10.1007/s10753-018-0922-4

ORIGINAL ARTICLE

MicroRNA let-7c Improves LPS-Induced Outcomes of Endometritis by Suppressing NF-κB Signaling

Gan Zhao,[1] Tao Zhang,[1] Haichong Wu,[1] Kangfeng Jiang,[1] Changwei Qiu,[1] and Ganzhen Deng[1,2]

Abstract— Endometritis is a common inflammatory disease which endangers human and animal reproductive health. MicroRNA (miRNA) let-7c plays an important role in the inflammatory process; however, the regulatory underlying mechanism of let-7c in endometritis is unclear. In this study, we confirmed that let-7c was significantly reduced in LPS-induced mouse endometritis model, and overexpression of let-7c was able to effectively reduce uterine tissue damage caused by lipopolysaccharide (LPS), and then, a LPS-induced bovine endometrial epithelial cell (BEND) line was used to mimic the inflammatory model *in vitro*. Our data showed that overexpression of let-7c significantly reduced the expression of pro-inflammatory cytokines in BEND cells induced by LPS. Meanwhile, immunofluorescence and western blotting results showed that let-7c significantly inhibited LPS-induced phosphorylation of NF-κB, thereby inhibiting downstream pro-inflammatory cytokine expression. Taken together, our results suggested that let-7c ameliorates LPS-induced endometritis by attenuating the expression of pro-inflammatory cytokines *via* inhibition of the activation of NF-κB.

KEY WORDS: let-7c; LPS; endometritis; nuclear factor-κB; pro-inflammatory cytokines.

INTRODUCTION

Endometritis is an inflammatory disease of the endometrial lining caused by many factors, such as bacterial invasion, endangering both animal and human health [1, 2].Gram-negative bacteria *Escherichia coli*(*E. coli*) has been identified as a major pathogenic microorganism leading to endometritis [3, 4]. It produces the endotoxin lipopolysaccharide (LPS) which is the major virulence factor of Gram-negative bacteria into uterine cavity and triggers severe inflammatory responses [5]. Thus, LPS has been often used to establish many inflammatory animal disease models, including endometritis [6, 7].

Nuclear factor (NF)-κB is a widely expressed nuclear transcription factor and is considered to be one of the most important regulators during inflammatory process [8, 9]. During the development of endometritis, LPS can cause neutrophil activation and induces the intracellular NF-κB signaling pathway activation; subsequently, it triggers the transcription of the expression of inflammatory genes and promotes the release of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), to aggravate inflammatory damage [2, 10]. Therefore, treatments aimed at inhibiting the activation of NF-κB may have potential therapeutic advantages for inflammatory diseases.

MicroRNAs (miRNAs) area class of non-coding small RNAs(~22-nt) involved in many cellular processes, including inflammation [11]. Let-7c is one of the most

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10753-018-0922-4) contains supplementary material, which is available to authorized users.

[1] Department of Clinical Veterinary Medicine, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, 430070, People's Republic of China

[2] To whom correspondence should be addressed at Department of Clinical Veterinary Medicine, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, 430070, People's Republic of China. E-mail: ganzhendeng@sohu.com

abundant and highly conserved miRNAs and has been implicated in mediating the inflammatory macrophage polarization and regulating immune responses against pathogenic stimuli [12, 13]. Iliopoulos et al. showed that Let-7/NF-κB signaling plays a significant role in the cancer-related inflammation [14]. Using let-7c mimic suppressed the release of inflammatory mediators and improves neurological outcomes in a traumatic brain injury mouse model [15]. Taken together, these reports indicate that let-7c may serve as a therapeutic potential for improving inflammatory diseases. Our previous study has suggested that let-7 regulated the inflammation balance during early pregnancy [2]; however, there is no experimental evidence to verify it. Therefore, we hypothesized that let-7c improves endometritis outcomes by suppressing NF-κB signaling. This study is aimed at determining the protective effects of let-7c on LPS-induced endometritis *in vivo* and *in vitro.*

MATERIALS AND METHODS

Cell Culture

BEND cells (bovine endometrial epithelial cells) were purchased from the American Type Culture Collection (ATCC CRL-2398™) and cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 (HyClone, USA) with 10% FBS (fetal bovine serum, PAN, Germany). BEND cells were treated with LPS (1 μg/ml, from *E. coli* O55:B5, Merck, Germany) alone or with other corresponding treatments. After the treatments, the cells were prepared for further studies.

Cell Transfection

The miRNA agomiRs were purchased from GenePharma Company (Shanghai, China). BEND cells were transfected with 50 nM miRNA agomiR or the control oligonucleotide (NC) in 6-well plates with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The whole transfection process was proceeded in a non-serum medium named opti-MEM (Gibco, Gaithersburg, MD, USA) for 6 h at 37 °C in a humidified environment containing 5% CO2. After transfection, the medium was changed into a previous medium and incubated for 18 h. After that, the LPS group (NC + LPS) and the agomiR treatment group (let-7c-5p), cells were treated with LPS (1 μg/ml) for 3 h, control group (NC) were treated with equal volume of PBS. Cells and cell supernatants were collected for further study.

RNA Extraction and qPCR

Total RNA was isolated from the uterine tissues and cells using TRIzol, according to the manufacturer's instructions (Invitrogen, USA). Then, cDNA was synthesized using a reverse transcription kit (Takara, Japan). Total cDNA was used as the starting material for real-time PCR with FastStart Universal SYBR Green Master Mix (Roche Applied Science, Germany). *GAPDH* served as an internal standard. The expression of miRNAs was confirmed using the stem-loop qRT-PCR method which describe as previously [2]. Briefly, Total RNAs from each samples were isolated by TRIzol, then reverse-transcribed into cDNA using a Reverse Transcriptase M-MLV (TaKaRa) and Hairpin-it™ microRNA qPCR Quantitation Kit (GenePharma, Shanghai, China). qPCR was performed using a SYBR® Select Master Mix kit and standard protocols on a Step One Plus Real-Time PCR System (Applied Biosystems, USA). The $2-\Delta\Delta Ct$ method was used to analyze expression levels. The specific primers are listed in Supplemental Tables 1 and 2.

NF-κB p65 Immunofluorescence

Cells were cultured onto six-well chamber slides. During collecting, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.01% Triton X-100 for 10 min. Then, the cells were blocked for 1 h and treated with anti-pho-NF-κB p65 (#3033, Cell Signaling Technology, USA) at 4 °C overnight. Next, the cells were incubated with Cy3-conjugated Goat Anti-Rabbit IgG (Boster Biological Technology, Wuhan, China) for 60 min at RT. Nuclei were stained using 4′,6-diamidino-2-phenylindole (DAPI). Fluorescent images were taken using an AX70 wide field microscope (Olympus). All morphometric measurements were performed by at least three independent individuals in a blinded manner.

Cytokine Assays

Cytokine levels (IL-1β, TNF-α, and IL-6) in cell supernatants were measured using ELISA kits according to the manufacturer's instructions (Biolegend, USA). The absorbance was detected at 450 nm with a microplate reader (Thermo Scientific Multiskan MK3, USA).

Mouse Model and Sampling

Six- to 8-week-old Kunming (KM) mice were obtained from the Animal Experiment Center of Huazhong Agricultural University (Wuhan, China) and housed at a constant temperature (23 °C) and relative humidity (60%) with a fixed12-h light:12-h dark cycle with free access to food and water. All experimental procedures involving animals and their care were approved by the Animal Welfare and Research Ethics Committee of Huazhong Agricultural University. The LPS-induced endometritis mouse model was developed as previously described [10]. Briefly, mice were administered equal amounts of LPS (1 mg/kg) on each side of the uterus under anesthesia, and the control group received equal volumes of a sterile saline solution. Mice of each group were treated as indicated above and then killed *via* CO2 inhalation. Uterine tissues from each group were harvested and immersed in 4% paraformaldehyde for H&E staining. The remaining tissues were stored at − 80 °C for subsequent experiments.

Histopathological Analysis

The uterus tissues were embedded in paraffin, sectioned, dehydrated, and then cut into 5-μm-thick sections for hematoxylin and eosin (H&E) staining. Finally, the sections were observed using an optical microscope (Olympus, Japan).

MPO Activity Assay

The uterus tissues (weighing approximately 50 mg) were homogenized with reaction buffer, and the MPO activity was detected by the MPO Assay Kit (Jiancheng biotechnology, China) according to the manufacturer's instructions and measured with a spectrophotometer at wavelength of 460 nm.

Western Blotting

The total protein of uterus tissues and cells were extracted, and the concentration of proteins was determined using a BCA kit (Vazyme, China). Western blot analysis was performed as previously described [8]. Briefly, samples with equal amounts of protein (50 μg) were fractionated on 10% SDS–polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and blocked in 5% skim milk in TBST for 1.5 h at 25 ± 1 °C. The membranes were then incubated at 4 °C overnight with 1:1000 dilutions (*v*/*v*) of the primary antibodies. After washing the membranes with TBST, incubations with 1:4000 dilutions (*v*/*v*) of secondary antibodies were conducted for 2 h at 25 ± 1 °C. Protein expression was detected using an enhanced chemiluminescence detection system (ImageQuant LAS 4000 mini, USA). β-Actin was used as the control.

Statistical Analysis

All statistical analyses in this study were performed with GraphPad Prism 5 (GraphPad InStat Software, CA, USA). The data are expressed as the means ± standard error of the mean (S.E.M.). Data between two groups were compared with unpaired two-tailed Student's *t* test. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Altered let-7c-5p Expression in Uterus After LPS Induced Injury

We detected let-7c-5p expression levels in uterus at different time points from 0 to 72 h post-injury as measured by qPCR (Fig. 1). The expression of let-7c-5p in the LPS group was reduced after injury, reached a plateau on 24 h post-injury then gradually increased to baseline at 72 h post-injury. These data indicated that LPS led to downregulation of let-7c-5p in the uterus, and upregulation of let-7c-5p may protect against uterine injury.

Upregulation of let-7c-5p Level in Uterus Improves the Uterine Pathological Outcomes Induced by LPS

To verify the protective effect of let-7c-5p *in vivo*, we treated mice ($n = 5$) with let-7c-5p mimic (agomiR) at 12 h, 36 h post-injury (i.p., 0.5 μmol/kg) and sampled at 48 h post-injury. Compared with the mimic control (NC) group, the let-7c-5p expression level was significantly increased in the mimic group (LPS + let-7c-5p) (Fig. 2a), accompanied by a marked protection from LPS-induced

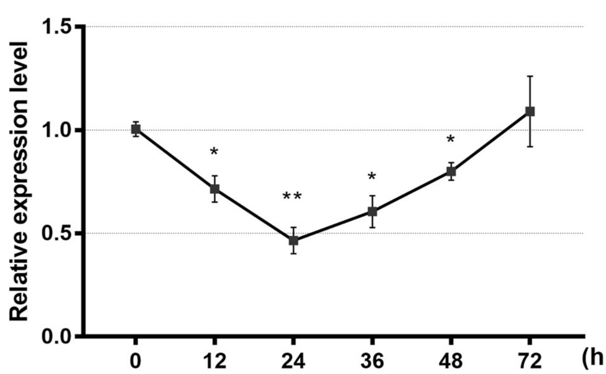

Fig. 1. Altered let-7c-5p expression level in the uterus after LPS-induced injury. Data are presented as the mean ± S.E.M ($n = 5$). Two-tailed unpaired Student's *t* test, $*P < 0.05$; $**P < 0.01$.

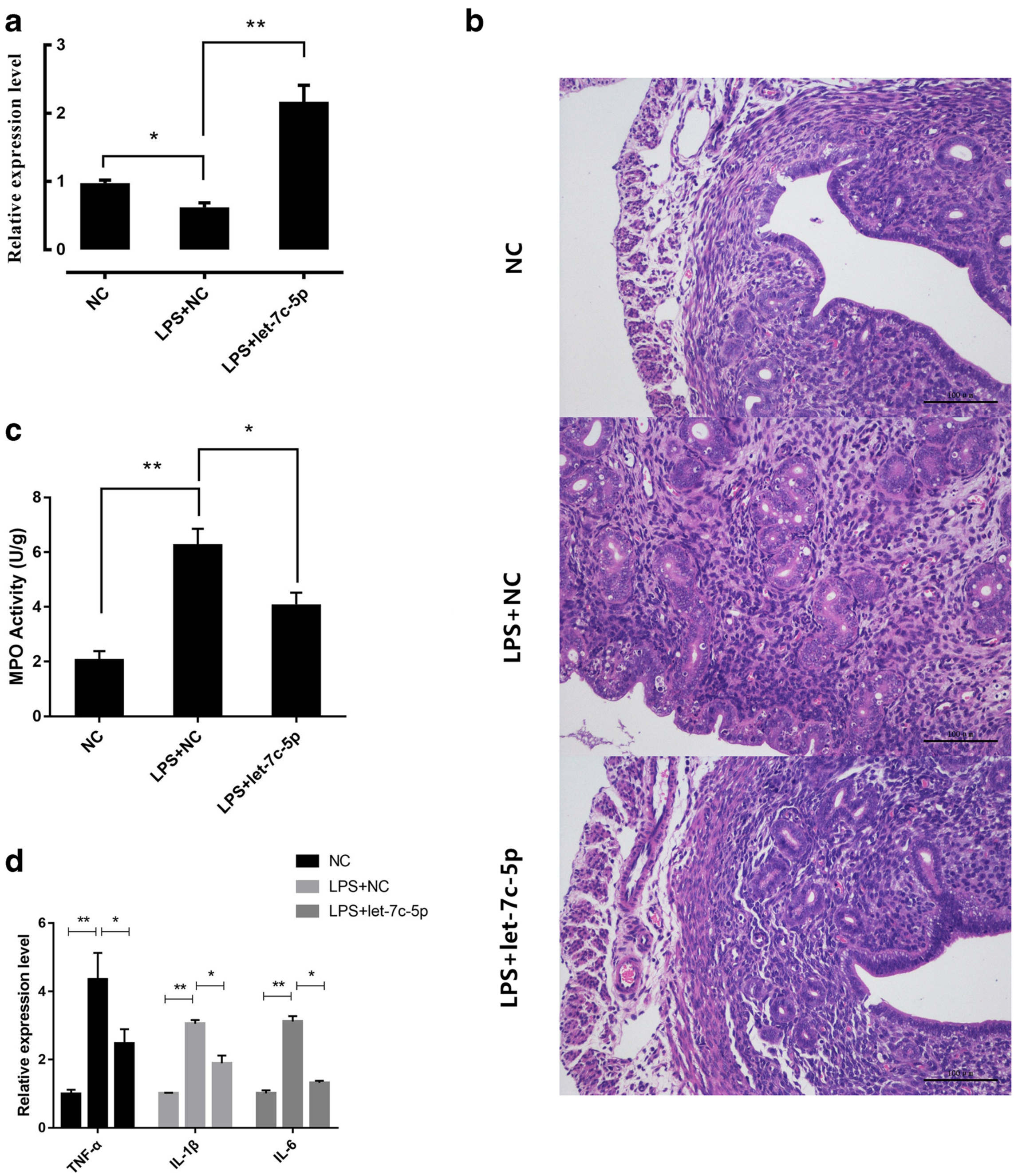

Fig. 2. Upregulation of let-7c-5p level in the uterus improves LPS-induced endometritis. **a** let-7c-5p expression was measured in uterine tissue by qPCR. **b** H&E staining of uterine tissue, scale bar, 100 μm. **c** MPO activity. **d** Relative mRNA of TNF-α, IL-1β, and IL-6 was assessed by qPCR in whole uterine biopsies; $n = 3–5$ mice/group. Data represent three independent experiments and are presented as the mean ± S.E.M (error bars). Statistical significance determined by unpaired Student's t test.

endometritis, as indicated by the attenuated pathology conditions and MPO activity (Fig. 2b, c). Furthermore, let-7c-5p mimic treatment repressed the mRNA levels of IL-1β, IL-6, TNF-α (Fig. 2d). Collectively, these data confirmed the pivotal role of let-7c-5p for the treatment of experimental endometritis.

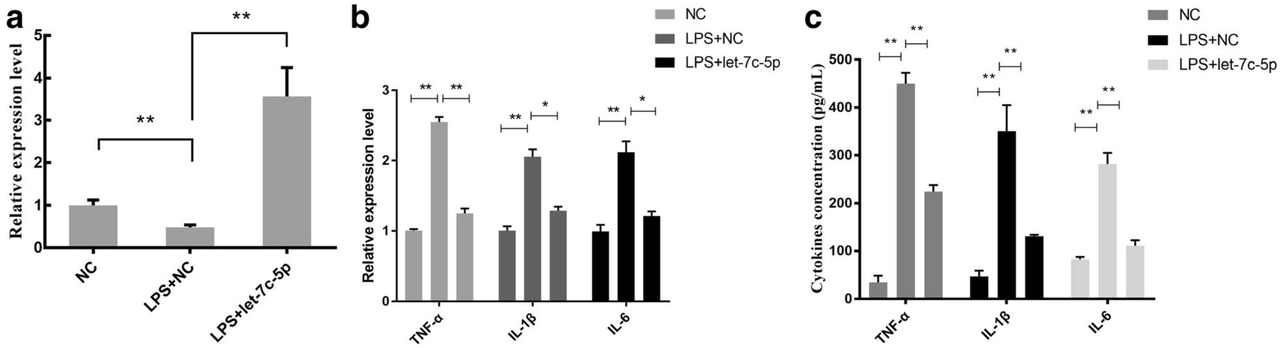

Fig. 3. Overexpression of let-7c-5p attenuates LPS-induced pro-inflammatory cytokines expression in BEND cell. **a** qPCR was used to detect let-7c-5p expression in BEND cells. **b**, **c** The pro-inflammatory cytokines IL-1β TNF-α, and IL-6 were detected by qPCR and ELISA. Data represent three independent experiments and are presented as the mean ± S.E.M (error bars). Two-tailed unpaired Student's *t* test, $^{*}P < 0.05$; $^{**}P < 0.01$.

Overexpression of let-7c-5p Attenuates pro-Inflammatory Cytokine Expression

BEND cells were transfected with let-7c-5p mimics (agomiR) for 24 h (Fig. 3a), followed by 3 h of exposure to LPS (1 μg/ml), and the pro-inflammatory cytokines IL-1β TNF-α and IL-6 were detected by qPCR and ELISA. The results showed that LPS challenge caused a significant increase in TNF-α, IL-1β, and IL-6. Compared with the LPS group, let-7c-5p mimic significantly reduced the levels of TNF-α, IL-1β, and IL-6 in both mRNA and protein levels (Fig. 3b, c).

let-7c-5p Mimic Treatment Regulates NF-κB Activation

NF-κB is the key regulator involved in inflammatory processes *via* regulating the expression of many inflammatory mediators. Previous study revealed that let-7c-5p regulated macrophage inflammatory phenotype reversion in an NF-κB-dependent pathway [13]. Thus, we determined the effects of let-7c-5p on NF-κB activation. Our results showed that phosphorylation of p65 significantly increased by 3 h of exposure to LPS (1 μg/ml); however, these values were decreased by let-7c-5p mimic treatment, which was also confirmed by the immunofluorescence assay. (Fig. 4a, b).

DISCUSSION

Endometritis brings a health threat to both humans and animals and poses a huge economic burden. Although antibiotics have been widely used to treat endometritis, the heavy use of antibiotics leads to the production of drug-resistant bacteria; therefore, it is urgent to develop new therapeutic strategies to treat endometritis. In the present study, we confirmed that let-7c-5p plays a protective effect by regulating the activation of NF-κB, thereby attenuating the release of pro-inflammatory cytokines during endometritis.

LPS acts as a primary infectious stimulus leading to severe inflammatory response**;** it can activate the NF-κB signaling [16]. Once NF-κB activated, the subunit p65 translocates from the cytoplasm to nucleus, which triggers the transcription and release of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6 [17]. In addition, these pro-inflammatory cytokines can induce the expression of other cytokines, leading to necrosis of epithelial cells and recruitment of neutrophils, thereby aggravating injury of the endometrial epithelium [18, 19]. Thus, inhibition of the release of inflammatory cytokines may be a target for anti-inflammatory drug therapies. MPO, known as a PMN (polymorph nuclear) marker enzyme, is used to as a direct measurement of the neutrophil granulocyte active state in tissues [19]. Our results which showed the MPO activity induced by LPS was significantly decreased by let-7c mimic treatment. This suggested that let-7c had a protective effect on LPS-stimulated endometritis.

Recently, the role of miRNA in inflammation receives increasing attention. Hailemariam and Salilew-Wondim et al. revealed a large number of miRNA profiles, including let-7c, associated with endometritis [20, 21]. However, their underlying molecular mechanisms remain unclear. Our data showed that the expression of let-7c was reduced after injury, reached a plateau on 24 h post-injury then gradually increased to baseline at 72 h post-injury. This change suggests that let-7c may be involved in tissue repair after LPS-induced injury. Ji et al. showed that let-7c functioned as a negative regulator of the inflammatory response

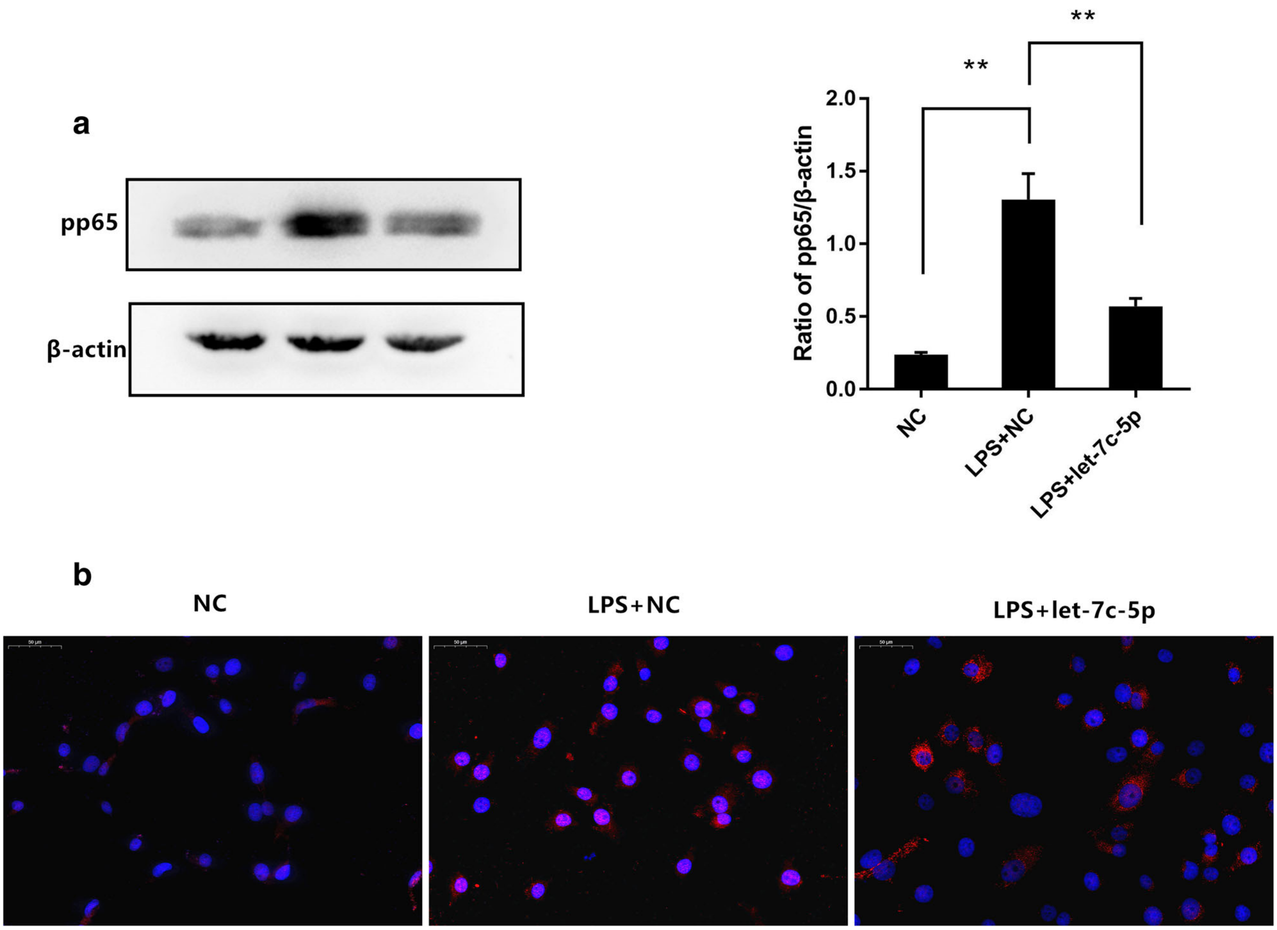

Fig. 4. Overexpression of let-7c-5p attenuates LPS-induced activation of NF-κB. **a** Western blot was performed to detect the phosphorylation level of NF-κB p65. **b** Translocation of the p65 subunit from the cytoplasm into the nucleus was evaluated by immunofluorescence. Blue spots represent cell nuclei, and red spots represent p-p65 staining; scale bar 50 μm. Data represent three independent experiments and are presented as the mean ± S.E.M (error bars). Two-tailed unpaired Student's *t* test, $*P < 0.05$; $**P < 0.01$.

in a chronic obstructive pulmonary disease (COPD) in mouse model by decreasing the release of pro-inflammatory cytokines [22]. NF-κB serves as a target in many inflammatory diseases due to its ability to modulate the expression of downstream pro-inflammatory cytokines. Therefore, inhibiting its activation can control the development of endometritis. Our results showed that overexpression of miRNA let-7c-5p attenuated the expression of TNF-α, IL-1β, and IL-6 by suppressing the NF-κB. Since let-7c does not directly inhibit NF-κB activation, there may be a target of let-7c acting as an activator of NF-κB, such as Ras, which is inhibited by let-7c and blocks NF-κB activation [23, 24]. However, this study focused on the indirect inhibition of NF-κB by let-7c to attenuate the expression of downstream pro-inflammatory cytokines and exert anti-inflammatory protective effects.

Collectively, our study highlights that miRNA let-7c serves as a protective factor in the inflammatory response. Thus, let-7c may hold promise as a future novel therapeutic modality for active flares of endometritis and other inflammatory diseases.

ACKNOWLEDGEMENTS

Thank all members in the Laboratory of Veterinary Clinical Diagnosis with all the helpful suggestions. We especially thank Dr. Aftab Shaukat for English language editing.

Funding Information

This work was supported by the National Natural Science Foundation of China (Grant No. 31772816).

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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