ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
In press.

The Mechanism of Notopterol Alleviating LPS-induced Endometritis by Inhibiting the TLR4/NF-κB Signaling Pathway

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Received: 20 November 2024; Received in revised form: 15 January 2025; Accepted: 13 February 2025

ABSTRACT

This study aims to investigate the role of notopterol in alleviating endometritis induced by lipopolysaccharide (LPS) and to explore its underlying mechanisms.Human endometrial epithelial cells (hEECs) were treated with LPS to establish an *in vitro* model of endometritis, and the cells were divided into five groups: control, LPS, LPS+notopterol(15 mol/L), LPS+notopterol(305 mol/L) and LPS+notopterol(45 mol/L) groups. The expression levels of inflammatory factors were determined by Enzyme-Linked ImmunoSorbent Assay (ELISA). Apoptosis was detected by TdT-mediated dUTP Nick-End Labeling (TUNEL) method. Cell viability was determined by Cell Counting Kit-8 (CCK-8) test. Western blot was used to detect the expression levels of nuclear factor ϰB(NF-ϰB) p65, NF-ϰB inhibitor (IϰBα), p-NF-ϰB p65 and p-IϰBα.

Following LPS treatment, cytokine levels significantly increased compared to the control group.; moreover, cell proliferation decreased, apoptosis increased, and the expression level of p-NF-ϰB p65 was increased. Subsequently, the LPS-treated hEECs were exposed to notopterygium. Compared to the LPS group.

Treatment with LPS + notopterol resulted in a dose-dependent reduction in inflammatory cytokines, increased cell proliferation, and a significant reduction in apoptosis. Furthermore, the expression levels of p-NF-ϰB p65 and p-IϰBα were downregulated.

These findings suggest that notopterol alleviates LPS-induced endometritis by inhibiting the TLR4/NF-ϰB signaling pathway.

Keywords: Endometritis; Inflammation; Lipopolysaccharide; Notopterol

INTRODUCTION

Endometritis is an inflammatory disease caused by various pathogenic microorganisms.[1] It primarily affects sexually active women of reproductive age, and is rare in prepubertal, sexually inactive, and postmenopausal women. Mild cases may present with no obvious symptoms or only lower abdominal pain or increased vaginal discharge. In contrast, severe cases may involve fever or symptoms affecting the digestive and urinary systems. If not treated in time, endometritis can progressto chronic pelvic inflammatory conditions, such as low back pain and pelvic pain, and may cause ectopic pregnancy, infertility, and other complications that severely affect patients' physical and mental health.[2] Antibiotics are the most commonly used treatment for

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endometritis. However, prolonged antibiotic use can promote bacterial resistance and hepatorenal toxicity.[3-5] Additionally, the overuse of antibiotic can compromise the uterus's ability to defend against bacterial infections.[5,6] These challenges highlight the urgent need to develop new drugs for endometritis treatment.

The Toll-like receptor 4 (TLR4)/myeloid differentiation factor 88 (MyD88)/nuclear factor-κB (NF-κB) signaling pathway is a classic pathway that mediates inflammatory responses. Under normal conditions, NF-κB is anchored to the cytoplasm. However, upon receiving inflammatory signals, NF-κB becomes activated by MyD88 and undergoes nuclear translocation, thereby regulating the expression of related proinflammatory factors and inducing inflammation. Numerous studies have confirmed that MyD88 acts as a downstream signaling molecule regulated by *TLR4*.[7-9] Research have found that TLR4 is highly expressed in endometrial cells, decidual cells and trophoblast cells, and is involved in inflammatory reactions, immune regulation and uterine pregnancy processes.[5,10] Endometrial epithelial cells (EECs) are the first line of defense in the endometrium, and express most TLR genes.[5,11] During the pathogenesis of endometritis, lipopolysaccharide (LPS) recognizes *TLR4* receptors on the surface of endometrial epithelial cells and binds to specific structural sites of *TLR4*, therby activating the NF-κB signaling pathway.[5,12] After the NF-κB signaling pathway is activated, NF-κB inhibitor (IκBα) is phosphorylated and degraded; the NF-κB p65 subunit is phosphorylated and transferred from the cytoplasm to the nucleus, inducing the release of various proinflammatory factors including Tumor Necrosis Factor-alpha (TNF-α), Interleukin-1 beta (IL-1β), Interleukin-6 (IL-6), thereby aggravating the inflammatory response.

Notopterygium (Umbelliferae) is a perennial herbaceous plant that belongs to the legume family. The main medicinal parts are the roots and rhizomes. Notopterygium is mainly distributed in the Shaanxi, Sichuan, Qinghai, Gansu, and Yunnan provinces of China. It is considered bitter and warm acting on the meridians of the bladder and kidneys. Clinically, it is mainly used to treat wind-cold colds, cold-damp arthralgia, neck stiffness and tightness, bone and joint pain, and anasarca caused by wind and folliculitis.[13] Pharmacological studies have shown that Notopterygium exerts anti-inflammatory, analgesic, anti-myocardial ischemia, anti-shock, antithrombotic, and antioxidant properties.[14-17] Their anti-inflammatory effects are particularly notable. Notopterol, the main active ingredient in Notopterygium, plays a significant role in its anti-inflammatory effects. However, its anti-endometric effect and mechanism of action remain unclear and require further investigation.

Based on the background described above, we hypothesized that notopterol regulates the TLR4/NF-κB signaling pathway, inhibits NF-κB signaling, and alleviates LPS-induced cell inflammatory damage. Using human endometrial epithelial cells (hEECs) to establish a model of endotoxin-induced endometritis, we intervened with notopterol and explored the mechanism by which notopterol alleviates LPS-induced endometritis through TLR4/NF-κB signaling.

MATERIALS AND METHODS

Cells and Drugs

hEECs were purchased from Shanghai Yuchun Biology Science and Technology Co., Ltd. and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fatal Bovine Serum (FBS) at 37°C and 5% CO_2.[18] Notopterol was obtained from Chengdu Herbpurify Biotechnology Co., Ltd.(City: Chengdu; Street:Room709-C1,Incubator Building, Tianfu Life Science Park No.88,Keyuan Road, Gaoxin District, Chengdu, Sichuan, China) and LPS was sourced from Shanghai Regal Biotechnology Development Co., Ltd (City: Shanghai; Street: 4D60-4, No. 1611, Caoan Road, Jiading District, Shanghai, China).

Reagents and Instruments

Cell lysis buffer (P0013, Beyotime, Shanghai, China), human TNF-α ELISA Kit (PT518, Beyotime, Shanghai, China), human IL-1β ELISA Kit (PI305, Beyotime, Shanghai, China), and human IL-6 ELISA Kit (PI330, Beyotime, Shanghai, China), the SDS-PAGE gel preparation kit (P0012AC, Beyotime, Shanghai, China) and 5× protein loading buffer (P0283, Beyotime, Shanghai, China) were sourced from Beyotime Biotechnology Co., Ltd. The Bicinchoninic Acid (BCA) protein quantitation assay kit (A045-4-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was obtained from Nanjing Jiancheng Bioengineering Institute. Phosphate buffered saline (PBS) (ZLI-9061, Zhongshan Golden Bridge, Beijing, China) was sourced from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. The PVDF membrane

(10600069, GE Healthcare, Buckinghamshire, UK), enhanced chemiluminescence (ECL)-Plus chemiluminescence reagent (RPN2106, GE Healthcare, Buckinghamshire, UK) and high-performance autoradiography film were supplied by Amersham. The developer (FT-LG1084, Kodak, New York, USA) and rapid fixer (67-68-5, Kodak, New York, USA) were purchased from the Kodak Company. A CCK-8 kit (BL1055B, Sailesi, Chengdu, China) was sourced from Chengdu Sailesi Technology Co., Ltd. ELISA Kit (GH00325, Genehunter, Nanjing, China) was purchased from Nanjing Manforit Technology Co., Ltd. The p-IκBα antibody (EY-01K5575, C-reagent, Shanghai, China) was purchased from Shanghai C-reagent Biotechnology Co. Ltd. The p-NF-κB p65 antibody (sc-33020, Fusheng, Shanghai, China) was obtained from Shanghai Fusheng Industrial Co., Ltd. A CytoFLEX flow cytometer was purchased from Beckman Coulter, Inc. A TGL-16G low-speed desktop centrifuge was acquired from the Shanghai Anting Scientific Instrument Factory.

Experimental Groups

hEECs were cultured in a complete medium containing 5% FBS, 1% epithelial cell growth factor, and 1% penicillin and streptomycin. Cells were treated with LPS (10 μg/mL) to establish an in vitro cell inflammation model. The experiment included 5 groups: control, LPS (10 μg/mL) group, LPS + notopterol (15 μmol/L) group, LPS + notopterol (30 μmol/L) group, LPS + notopterol (45 μmol/L). Samples were collected 24 h after modeling.[19]

ELISA to Detect TNF-α, IL-1β and IL-6 Levels in hEEC Supernatant

Firstly, the well-grown hEECs were seeded into a 12-well plate at a density of 1×10^5/mL.[20] After drug treatment, the cells were further cultured, and the supernatant was collected and centrifuged after 12 hours. Secondly, the standard and samples were added into a 96-well plate coated with corresponding antibody in advance, and washed after incubation. Finally, the substrate chromogenic solution was added, and after incubation, the stop solution was added, then the absorbance value was read at a specific wavelength by enzyme-labeled instrument. The minimum detection limits were 14.3 pg/mL for TNF-α, 2.2 pg/mL for IL-1β, and 4.75 pg/mL for IL-6. The concentrations of TNF-α, IL-1β, and IL-6 in hEEC supernatant were calculated by comparing the sample's absorbance to that of the standard curve.[19]

TUNEL Staining Methods to Detect hEEC Apoptosis

Cells were seededinto a 12-well plate, and inoculated into the culture wells at a density of 1×10^5 cells, and theywere cultured until the cells adhered to the wall. After drug treatment, the cells were washed twice with preheated PBS, fixed with 4% paraformaldehyde for 2 hours, incubated with TUNEL staining solution at room temperature for 2 hours, and finally stained with DAPI in the dark at 4℃ for 30 minutes. The cells were then observed and photographed under a fluorescent microscope.[21]

CCK-8 to Detect Cell Viability

Well-grown hEECs were seeded into 96-well plates at a density of 2×10^4 cells/mL. After 24 hours of culture, 100 μL of LPS (10 μg/mL) was added to each well. At 0, 12, 24, 48, and 72 hours post-treatment, the old medium was discarded, 10 μL CCK-8 reagent was added to each well and incubated for 1hour. The optical density (OD) was measured at 450 nm to determine cell viability. The same procedure was repeated after notopterol treatment. Cell viability (%) =[A (with drug)–A (blank)]/[A (without drug)–A (blank)]×100%[22].

Western Blot to Detect IκBα, p-IκBα, NF-κB p65 and p-NF-κB p65 Levels

After drug treatment, the cells were washed 3 times with pre-cooled PBS and lysed with RIPA buffer for 30 minutes. The lysate was transferred to 1.5 mL EP tubes, centrifuged at 12000 rpm for 10 min, and the supernatant was collected into 1.5 mL centrifuge tubes and stored at -20°C. There were 3 samples in each group. After BCA protein quantification, the proteins were proportionally added to the protein-loading buffer and boiled at high temperature for later use. Gel preparation, electrophoresis, membrane transfer, and blocking were performed. The membranes were then incubated with primary antibodies against GAPDH (1:5000), IκBα (1:1000), p-IκBα (1:1000), NF-κB p65 (1:1000), p-NF-κB p65 (1:1000) respectively, and incubated overnight at 4℃.[23] The following day, membranes were incubated with the corresponding secondary antibody (1:10000) for 2 hours at room temperature. After washing, the the developer reagent was evenly dripped onto the strips, and the membrabne were imaged.

Statistics

Data were processed using SPSS 24.0. Continuous data are expressed as "mean ± standard deviation". One-way ANOVA was used for multiple comparisons. The LSD test was used for pairwise comparisons when variances were equal. Dunnett's test was used when variances were unequal. Statistical significance was set at $p<0.05$. difference. Images were processed using ImageJ 8.0.

RESULTS

LPS Treatment Had no Obvious Effect on the Morphology of hEECs

First, the morphological changes of hEECs treated with LPS were examined. We observed that the cells floated more after LPS treatment, but their overall morphology did not change significantly (Figure 1).

LPS Induces the Release of Cytokines in hEECs.

The expression levels of inflammatory factors in hEECs were measured by ELISA. As shown in Figure 2, the levels of cytokines TNF-α, IL-1β and IL-6 increased significantly after LPS treatment ($p<0.05$), indicating that LPS, induced a cellular inflammatory response (Figure 2).

LPS Inhibits the Proliferation and Promotes Apoptosis of hEECs.

Furthermore, the proliferation and apoptosis of hEEC*s* under different treatments were analyzed. Compared to the control group cell proliferation was significantly reduced after LPS treatment (Figure 3A, $p<0.05$). Simultaneously, LPS treatment significantly elevated apoptosisin hEEC. (Figure 3B, $p<0.05$).

Effect of LPS Treatment on NF-κB p65 Expression in hEECs

The effect of LPS treatment on NF-κB p65 expression in hEECs was evaluated. We found that the expression of p-NF-κB p65 was significantly upregulated in hEECs following LPS treatment (Figure 4, $p<0.05$).

Notopterol Inhibits the Release of Inflammatory Factors from hEECs Induced by Lipopolysaccharide

To investigatethe effects of notopterol on cell inflammation, LPS-induced cells were treated with different concentrations of notopterol (15 mmol/L, 30 mmol/L and 45 mmol/L). The results showed that the levels of TNF-α, IL-1β and IL-6 decreased as the concentration of notopterol increased (Figure 5, $p<0.05$), These findings suggest that notopterol can attenuate the levels of TNF-α, IL-1β and IL-6 in hEECs that induced by LPS in a concentration-dependent manner.

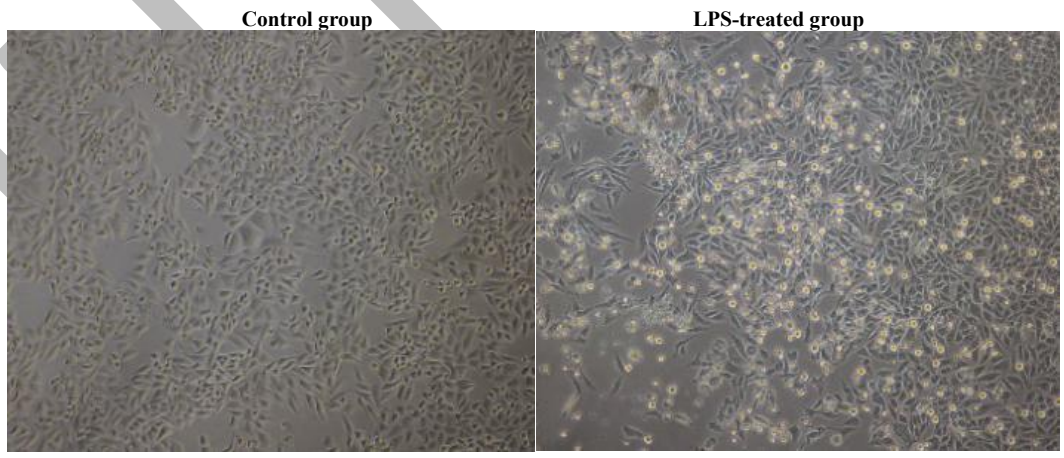

Figure 1. Effect of Lipopolysaccharide (LPS) treatment on cell morphology.

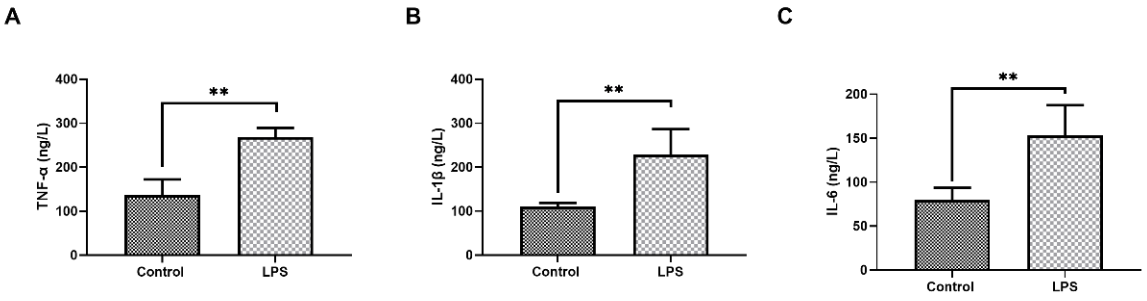

Figure 2. Changes of cytokines Tumour Necrosis Factor alpha (TNF-α), interleukin-1beta (IL-1β) and interleukin-6 (IL-6) in cells after LPS treatment. A: The release of TNF-α was detected by Enzyme-Linked Immunosorbent Assay (ELISA); The release of IL-1β was detected by ELISA; The release of IL-6 was detected by ELISA. *p<0.05, **p<0.01.

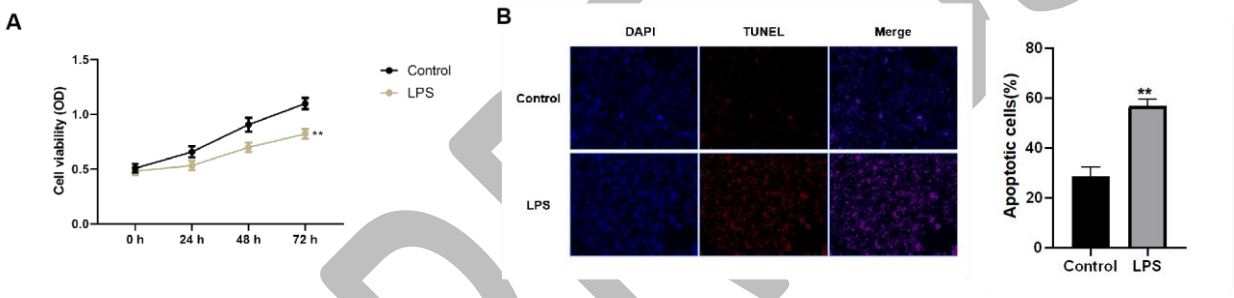

Figure 3. Changes of cell proliferation and apoptosis after LPS treatment. A: Cell Counting Kit-8 (CCK-8) to detect cell proliferation; B: Terminal Deoxynucleotidyl Transferase Mediated dUTP Nick End Labeling (TUNEL) staining methods to detect human endometrial epithelial cells (hEECs)apoptosis. *p<0.05, **p<0.01.

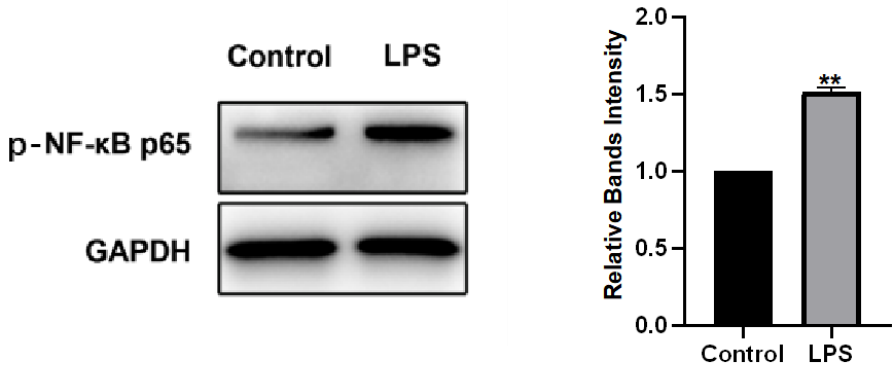

Figure 4. Expression of phosphorylated -nuclear factor kappa B p65 (p-NF-κB p65)p65 in cells after LPS treatment detected by Western blot and then perform grayscale quantization using Image J.

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Notopterol Promotes the Proliferation and Inhibits Apoptosis of hEECs

To investigate the effects of notopterol on proliferation and apoptosis of LPS-treated cells, we treated cells with different concentrations of notopterol (15 mmol/L, 30 mmol/L and 45 mmol/L) following LPS treatment, establishing an *in vitro* inflammatory model. The results demonstrated that compared to the LPS group, increasing concentration of notopterol improved cellproliferation (Figure 6A, $p<0.05$) and reduced apoptosis (Figure 6B, $p<0.05$). These results indicate that notopterol can can dose-dependently mitigate the inhibitory effect of LPS on cell proliferation and apoptosis Induction.

Notopterol Can Alleviate the Phosphorylation of NF-κB Subunit p65 Induced by Lipopolysaccharide and Its Transfer from Cytoplasm to Nucleus

In order to further explore the effects of notopterol on TLR4/NF-κB pathway, we performed WB analysis. The results showed that the expression of p-NF-κB p65 in hEECs increased significantly after LPS treatment, while the phosphorylation level of NF-κB p65 in IκBα decreased after notopterol treatment (Figure 7, $p<0.05$).

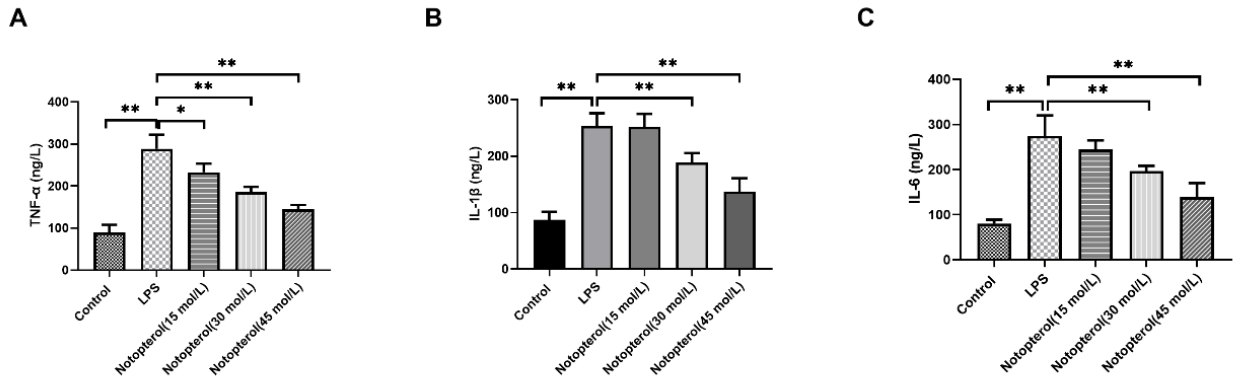

Figure 5. Changes of cytokines TNF-α, IL-1β and IL-6 in LPS-induced cells treated with notopterol. A: Release of TNF-α; B: Release of IL-1β; C: Release of IL-6. *$p<0.05$, **$p<0.01$.

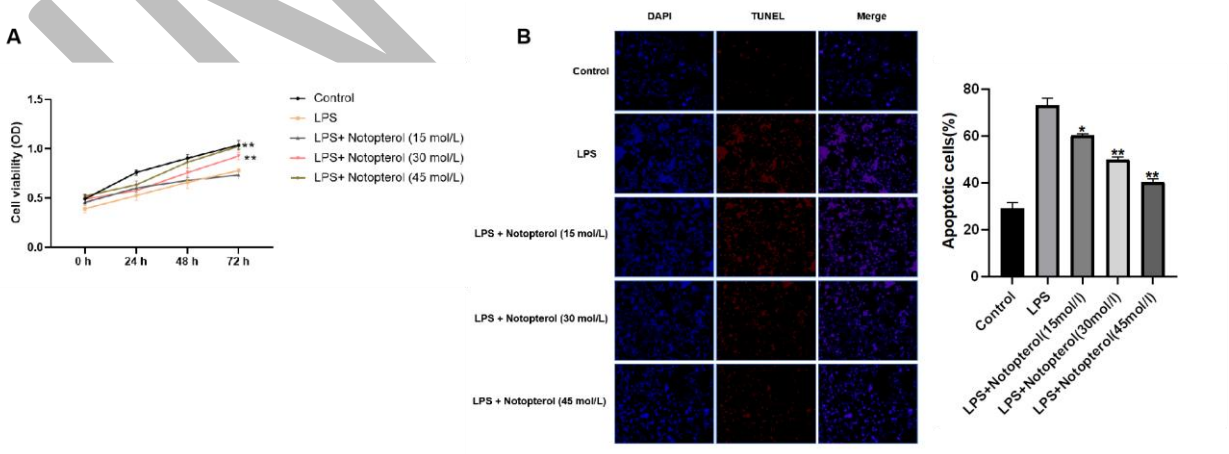

Figure 6. Changes of cell proliferation activity and apoptosis after treatment with novotopterol. A: CCK-8 to detect cell proliferation; B: TUNEL staining methods to detect hEECs apoptosis. *$p<0.05$, **$p<0.01$.

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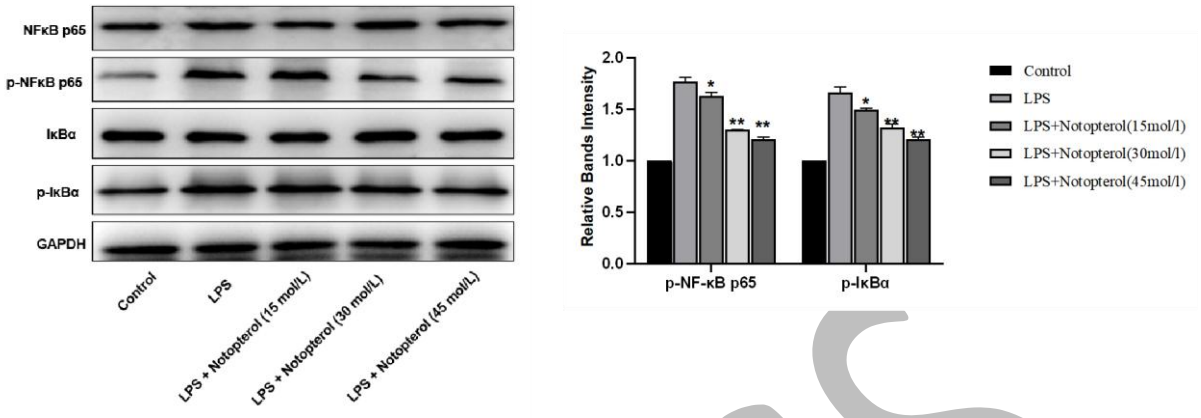

Figure 7. Changes of p-NF-κB p65 and phosphorylated inhibitor of kappa B alpha (p-IκBα) expression after notopterol treatment through Western blot.

DISCUSSION

Endometritis is a common uterine inflammation typically caused by bacterial infection, poor personal hygiene, immune dysfunction, or hormonal imbalances.[5,24] Studies have shown that bacterial infection is the most common pathogenic factor, with menstrual disorders, irregular vaginal bleeding, and infertility as the main clinical manifestations.[5,25] LPS , a structural component on bacterial cell walls, induces immune responses and inflammation, making it a suitable agent for establishing to an endometritis model.

LPS binds to *TLR4* receptors on the surface of hEECs, activating inflammatory signaling pathways within cells, leading to the secretion of proinflammatory cytokines such as TNF-α, IL-1β, IL-6 into the extracellular space, contributing to the occurrence and development of inflammation.[26,27] The TLR4/NF-κB signaling pathway is a classic inflammatory response pathway. The NF-κB pathway is activated when LPS binds to specific sites on *TLR4* on the cell surface. Following NF-κB activation, IκBα becomes phosphorylated and degraded, then the NF-κB p65 subunit is phosphorylated and transferred from the cytoplasm to the nucleus. In the current study, LPS-stimulated cells induced by LPS were treated with varying concentrations of notopterol. Comparing to the LPS group, the levels of TNF-α, IL-1β, IL-6, p-NF-κB p65 and p-IκBα, as well as cell viability and proliferation were detected to explore the mechanism how notopterol alleviates LPS-induced endometritis through the TLR4/NF-κB signaling pathway.

In this study, the levels of TNF-α, IL-1β, and IL-6 were significantly increased ($P<0.05$) in the cells after LPS treatment, followed by the treatment of LPS-induced cells with notopterol. The results showed that notopterol treatment was able to reduce the LPS-induced elevation of cytokine TNF-α, IL-1β, and IL-6 content in a concentration-dependent manner. The proliferative ability of the cells was significantly decreased after LPS treatment, while the proliferative ability of the cells was restored after notopterol treatment. It was suggested that notopterol could alleviate LPS-induced inhibition of cell proliferation activity by LPS. Cell proliferation was increased after LPS treatment, whereas apoptosis decreased under notopterol treatment, suggesting that notopterol alleviated LPS-induced apoptosis. The expression of p-NF-κB p65 was significantly increased in LPS-treated cells, whereas the phosphorylation level of NF-κB p65 with IκBα was decreased after notopterol treatment. It is suggested that notopterol can alleviate the LPS-induced transfer of NF-κB p65 subunit phosphorylation from the cytoplasm to the nucleus. Notopterol has been suggested to alleviate LPS-induced cellular inflammatory injury by inhibiting NF-κB signaling. Previous studies have been shown that Notopterol inhibits osteoclast production and prevents bone loss caused by ovariectomy by inhibiting NF-kB and NFATc1 signaling pathways induced by NF-kB ligand receptor activator (RANKL) and activating NF-E2 related factor 2 (Nrf2)/Keap1.[28] In addition, in osteoarthritis, Notopterol blocks the inflammatory corpuscles of NLRP3 through JAK2/NF-kB/hsa-miR-4282, results in alleviating the apoptosis triggered by Il

-1β. These suggest that Notopterygium alcohol may have effective anti-inflammatory and antioxidant characteristics. It is consistent with our research results.[29]

The Chinese Pharmacopoeia defines notopterygium as the roots and rhizomes of plants in the Umbelliferae family, notopterygium (Notopterygium incisum Ting ex H. T. Chang, NI) or broad-leaved notopterygium (N. franchetii H. de Boissieu, NF). It is classified into categories such as silkworm, bamboo, striped, and big-headed notopterygium according to its shape and quality. Notopterygium contains various complex components, including volatile oils, coumarins, enynes, sesquiterpenes, organic acids, organic acid esters, flavonoids, and sterols. Among these, coumarins are the main active ingredients in Notopterygium and are present at high levels.[13] Notopterols belong to the coumarins class of compounds. Modern pharmacological studies have shown that Notopterygium exhibits anti-inflammatory, analgesic, antipyretic, antithrombotic, antioxidant, antibacterial, antiviral, and antiarrhythmic properties.[13,17] Compounds such as falcarindiol, notopterol, nodakenin, isoimperatorin, and phenethyl ferulate have shown strong anti-inflammatory and analgesic activities.[30,31]

In summary, notopterol alleviates LPS-induced inflammatory damage by inhibiting the NF-κB signaling. A limitation of this study is that only a cell model was established. Future research will include validation through NF-κB pathway activator/inhibitor experiments and animal models to further confirm that notopterol can effectively alleviate LPS-induced cell inflammatory damage by inhibiting NF-κB signaling. This study offers new insights into the prevention and treatment of endometritis and supports the development of new drugs derived from traditional Chinese medicine.

STATEMENT OF ETHICS

Not applicable.

FUNDING

This research supported by Taizhou Science and Technology Plan Project (22ywb161); Zhejiang Province Traditional Chinese Medicine Science and Technology Plan Project (2025ZX367).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

Not applicable.

DATA AVAILABILITY

Upon reasonable request (Corresponding author: 591309812@qq.com)

AI ASSISTANCE DISCLOSURE

Not applicable.

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