Palmatine inhibits TRIF-dependent NF-κB pathway against inflammation induced by LPS in goat endometrial epithelial cells

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Abstract

Palmatine, a natural pharmaceutical drug, possesses many biological activities. But its clinical application is rarely reported in the veterinary medicine. The aim of this study was to investigate the anti-inflammatory effects of palmatine on lipopolysaccharide (LPS)-induced inflammation in goat endometrial epithelial cells (gEECs), and the possible molecular mechanisms. Palmatine cell toxicity was determined by MTT assay, and the production of inflammatory cytokine in the cultured medium was measured with ELISA, qRT-PCR and Western blotting. Our results showed that palmatine treatment inhibited the release of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, nitric oxide (NO), matrix metalloproteinase (MMP)-9 and MMP-2. Furthermore, palmatine enhanced the secretion of prostaglandins E_2 (PGE_2) and IL-10. Palmatine significantly down-regulated the expression of Toll-like receptor 4 (TLR4), cluster of differentiation 14 (CD14), Toll/interleukin 1 receptor (TIR)-domain-containing adaptor protein inducing interferon-β (TICAM, TRIF) and nuclear factor-κB (NF-κB) in LPS stimulated gEECs, but did not alter the production of MyD88. In conclusion, palmatine inhibits TRIF-dependent NF-κB pathway to reduce LPS-induced inflammatory responses in goat endometrial epithelial cells.

Key words

Palmatine, LPS, anti-inflammatory mechanism, TLR_4, NF-κB pathway

1. Introdution

Reproductive performance is a major concern in the livestock industry because of its

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important economic impact. Endometritis often leads to infertility, prolonged calving interval, reduced milk production, and increased treatment costs, which caused huge economic losses to the dairy farming industry [1]. In the past decades, the understandings of some important clinical disorders, including metritis, endometritis, and subclinical endometritis (SE), have advanced. It is reported that overall prevalence of cytological endometritis was 36.2% [2, 3]. Endometritis is considered a chronic inflammatory disease because mammals fail to completely clear postpartum bacterial contaminants in time [4].

The uterine immune response is generated not only by professional immune cells but also by endometrial epithelial and stromal cells. Endometrial cells express receptors that recognize microbe-associated molecular patterns (MAMPs), previously known as pathogen-associated molecular patterns (PAMPs). Lipopolysaccharide (LPS) is an important component of the outer membrane of Gram-negative bacteria and has been reported to be an important virulence factor. Bacteria which overwhelm the early mucosal defences in the genital tract activate an innate immune response by signaling through receptors such as Toll-like receptor (TLR)4 which recognizes bacterial LPS. This in turn triggers an inflammatory response involing an influx of inflammatory cells, a cascade of up-regulated inflammatory mediators (cytokines, chemokines, growth factors and lipid mediators) and tissue damage. In addition to immune cells, endometrial epithelial and stromal cells play crucial roles in uterine inflammation and immunity. The endometrium can regulate inflammatory response by releasing cytokines and chemokines [5]. Endometrial cells can respond to LPS through the Toll-like receptor (TLR)4 /CD14 /MD2 complex signaling pathway. They activate nuclear factor-κB (NF-κB) to regulate pro-infammatory genes expression and initiate inflammation [6, 7]. Once activated, these receptors trigger an inflammatory response aiming to eliminate the invading bacteria [8, 9].

Palmatine, a natural pharmaceutical drug, was listed in "pharmacopoeia of the People's

Republic of China" in 1977 [10]. It is a kind of quaternary amine isoquinoline alkaloid, with various biological activities. Clinically, palmatine is used as a remedy for abdominal pain, enteritis, gastritis, chronic endometritis, and pelvic inflammation [11, 12]. Also it has protective effects on acute and chronic inflammation in experimental animal models, such as ear edema, acetic acid induced capillary permeability test, and cotton pellet-induced inflammation [13,14]. Meanwhile, Wu et al demonstrated that palmatine can induce cell apoptosis to kill breast cancer cells MCF-7 [15]. Previous studies have reported its anti-inflammatory mechanisms. It reduced levels of inflammation in patients with acute episode of bronchial asthma by inhibiting the NF-κB activation, and relieved osteoarthritis via regulating the Wnt/β-catenin and Hedgehog signaling pathways [16, 17].

Compared with the above research, clinical application of Palmatine is rarely reported in veterinary. In this study, we assessed the anti-inflammatory effect of Palmatine on LPS-stimulated goat endometrium epithelial cells (gEECs), and investigated the possible singaling pathways of Palmatine involved endometritis.

2. Materials and methods

2.1. Reagents and antibodies

Palmatine (purity≥98%, Fig.1) was purchased from Shanghai source leaf Biological Technology Co., Ltd (Shanghai, China). LPS (Escherichia coli 0111:B4), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). DMEM-F12 and fetal bovine serum (FBS) were supplied by Gibco BRL (Invitrogen Corporation, Carlsbad, CA, USA). The enzyme-linked immunosorbent assay (ELISA) kits for goat of IL-1β, PGE_2 and IL-6 were purchased from Shanghai enzyme-linked Biological Technology Co., Ltd (Shanghai, China). TNF-α, MMP-9,

MMP-2 and IL-10 kits were purchased from Trust Specialty Zeal biological Trade Co. Ltd (Boston, MA, USA). Nitric oxide (NO) test kit was bought from the Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Anti-NF-κB p65 (ab131109, Abcam, USA) antibody was purchased from Abcam Biological Technology Co. (Cambridge, UK).

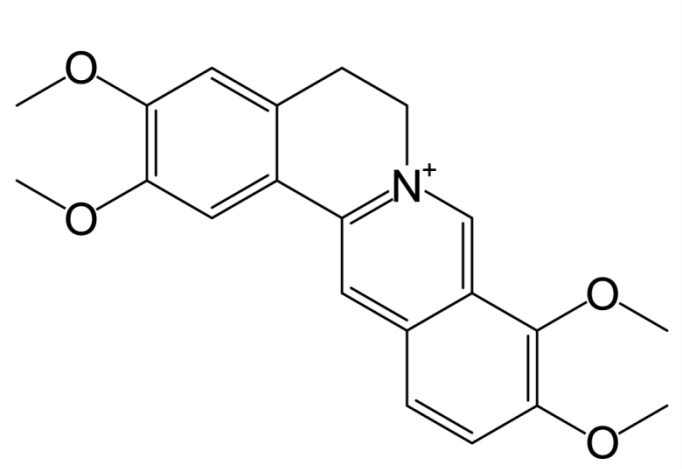

Fig.1. Chemical structure of Palmatine

2.2. Cell culture and cytotoxicity

Goat endometrium epithelial cells (gEECs) which are immortalized cells [18] were cultured at 37℃ and humidified with 5% CO2. They were cultured with the medium Dulbecco's modified eagle's medium-F12, DMEM-F12, containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin. GEECs were seeded into 96-well plate at a density of 1×10^4 cells/well with 0.2mL of the above culture medium for 24 h (control group), then exposed to different concentrations of Palmatine for 48 h. Viability of cells was estimated with an MTT assay [19, 20], then the inhibitory rate of cell proliferation was calculated as（A2－A0）/（A1－A0）×100%[21], where A_0 was the optical density of cells before the drug treatment; A_1 was the optical density of cells in control group and A_2 was the optical density of cells after the drug treatment for 48 h.

2.3. Cytokines assays in LPS-stimulated EECs

GEECs were seeded into 6-well plate at a density of 2×10^5 cells/well with the culture medium for 24 h (control group), exposed to LPS (5 μg/mL) for 12 h (LPS group), then treated with Palmatine at various concentrations (80, 40, 20 μg/mL) for additional 8 h. The levels of TNF-α, IL-1β, IL-6, PGE_2, MMP-9, MMP-2 and IL-10 in the supernatant were quantified using ELISA kits according to the manufacturer's instructions.

2.4. NO assays in LPS-stimulated EECs

The release of NO into supernatant of gEECs was spectrophotometrically determined by assaying thenitrite, a stable product of NO. Nitric oxide was detected using a Griess reaction. Absorbance was measured under 550 nm with a microplate reader, and sodium nitrite was used as a standard curve (Jiancheng Bioengineering Institute, Nanjing).

2.5. The mRNA expression determined by reverse transcription polymerase chain reaction

Total RNA was extracted with RNAiso Plus reagent (Takara, Dalian, China), then measured using 260/280 UV spectrophotometry. Equal quantities of RNA were reverse transcribed into cDNA using the Prime Script Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) according to the manufacturer's protocol. The primers were synthesized by Takara Biotechnology Co. (Dalian, China), and the primers sequences are given in Table 1. PCR reactions were set up with the SYBR®Premix Ex TaqTMII (Takara, Dalian, China) in BioRad Light cycler. After denature at 95℃for 30 s, PCR was performed with 40 cycles as follows: 95℃ for 5 s, 60℃ for 30 s and 55℃ for 20 s. All PCR experiments were performed in triplicates, including non-template controls. The relative levels of mRNA were calculated using the $2^{(-\Delta\Delta Ct)}$ method by comparing to GADPH.

Table 1 Primers used in real-time quantitative RT-PCR assays

Gene name	Primers Sequence（5′—3′）	Product size	Gene ID
TNF-α	ACGGCGTGGAGCTGAAA CTGATGGTGTGGGTGAGGAA	127bp	NM-001286442.1

IL-1β	TGAAGGCTCTCCACCTCCTCT TCTTGTTGTCTCTTTCCTCTCCTTG	90bp	D63351.1
TLR4	CTTGCGTCCAGGTTGTTCC CTCGGTTGATACGGGGATGT	89bp	NM_001285574.1
CD14	ACGACGATTTCCGCTGTGT ATACTGCTTCGGGTCGGTGT	155bp	XM_005683042.1
(RelA) NF-κB (p65)	CAGCTCACAGATCGGGAAAAG CGGTGCTGTCTGGAAGGAA	115 bp	JQ342.0881
MyD88	GGGACTAAGCGGAAGACCA GCTTCACCATTTCCCACGA	138bp	JQ308783.1
TICAM2	TTTCCTCTCAGTTTTCCTGGTTC TCGTTTTCTCCTGTGTGTCCTTT	150bp	JQ923482.1
GAPDH	AAGTTCCACGGCACAGTCAA ACCACATACTCAGCACCAGC	125bp	XM_005680968

2.6.The protein expression of NF-κBp65 by Western Blot

Collected gEECs after them were washed in phosphate-buffered saline (PBS, pH7.4). Total protein was extracted with a total protein extraction kit according to the manufacturer's instructions (Bestbio, Shanghai, china). The protein concentrations were determined by BCA protein assay kit (Takara, Tanapan). An equal amount of protein was loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel, then transferred to cellulose nitrate membrane. The membrane was blocked with 5% skim milk in Phosphate-buffered Saline containing 0.1% Tween 20 (PBS-T) for 2 h at room temperature, then incubated with the primary anti-body against NF-κBp65 (ab131109, Abcam, USA, 1:1000) for 2 h. After this, the membrane was washed with PBS-T at 5 min per time for five times. The membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (dilution 1:15,000), and washed 8 times with PBST. The immunoactive proteins were detected with an enhanced chemi-luminescence Western blotting detection kit (Takara, Dalian, China). Blots were normalized against β-actin to correct for differences in protein loading [22]. The result was analyzed using Image J gray level analysis software (Broken Symmetry software, UK).

2.7. Statistical analysis

Statistical data analysis was carried out using SAS 9.1 software (SAS Institute, NC, USA). Differences between groups were determined with one-way analysis of variance (ANOVA), followed by Duncan's multiple range test. Statistical differences between groups represented with (**) $P< 0.01$ vs. control group, ($^{\#\#}$) $P< 0.01$ vs. LPS group and ($^{\#}$) $P< 0.05$ vs. LPS group.

3. Results

3.1. Effect of palmatine on gEECs viability

Our results showed that palmatine at 10 ~ 227 μg/mL promoted the proliferation of gEECs. The effect of promoting proliferation was dose-dependent with a peak effect at 100μg/mL. However, when the concentration of palmatine in 100~ 227 μg/mL, the effect of promoting proliferation decreased with the increase of drug concentrations（Fig.2）.

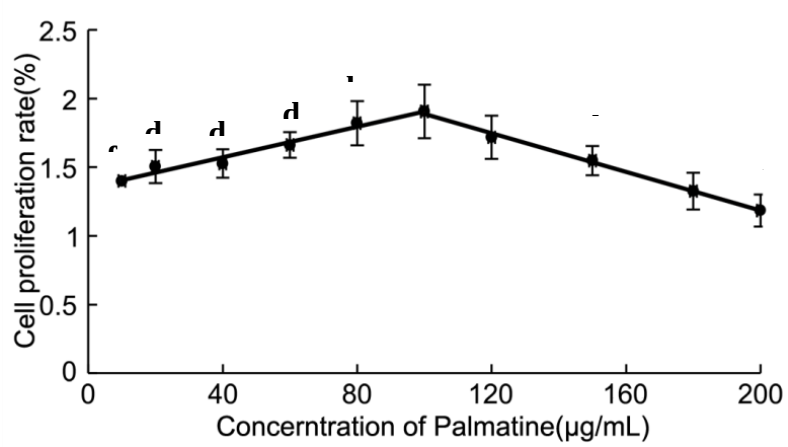

Fig.2. Effect of palmatine on the viability of gEECs. Cells were treated with palamtine for 48 h, and cytotoxicity was determined by the MTT assays. Values are expressed as mean ± SD (n=8).

3.2. Palamtine modulated inflammatory cytokines in LPS-stimulated EECs

The effects of palmatine on pro-inflammatory cytokines TNF-a, IL-1β, IL-6, and anti-inflammatory cytokine IL-10 were investigated in LPS-stimulated EECs. As depicted in Fig.3, LPS significantly increased the TNF-α, IL-1β, IL-6 and IL-10 levels in LPS group compared with the control group ($p < 0.05$). Palmatine treatment significantly inhibited the production of TNF-α, IL-6 and IL-1β in a dose-dependent manner compared with the LPS group ($P <$

0.05). It presented a different regularity about anti-inflammatory cytokine IL-10 from pro-inflammatory. With the 80 μg/mL palmatine treatment, the level of IL-10 decreased significantly, whereas palmatine at 40 and 20 μg/mL stimulated the secretion of IL-10 evidently ($P < 0.05$).

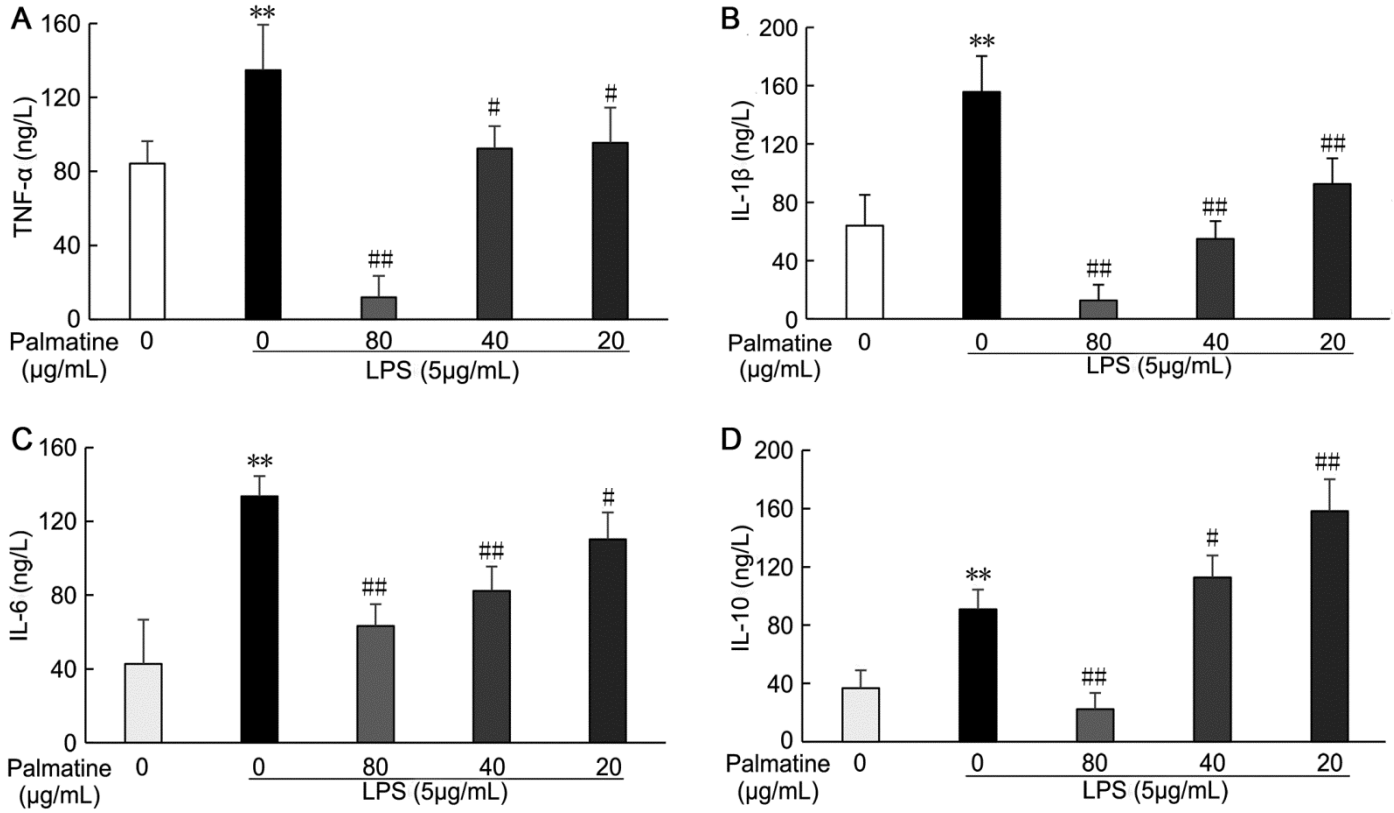

Fig.3. Modulatory effects of the palmatine (20, 40 and 80 μg/mL) on the level of TNF-a(A), IL-1β(B), IL-6(C) and IL-10(D) in LPS-stimulated gEECs. The cells were treated with the palmatine for 8 h after LPS administration. The control group was treated without palmatine and LPS. The concentrations of pro-inflammatory cytokines were determined with ELISA. Statistical differences between groups represented with (**) P< 0.01 vs. control group, (##) P< 0.01 vs. LPS group and (#) P< 0.05 vs. LPS group, values are expressed as mean ± SD (n = 3).

3.3. Palamtine modulated the production of PGE_2 and NO in LPS-stimulated EECs

As shown in Fig.4, LPS caused significant increase of NO and PGE_2 production compared with the control group ($P < 0.05$). Palmatine significantly inhibited the NO production in a dose-dependent manner. In contrast, palmatine significantly promoted the PGE_2 secretion.

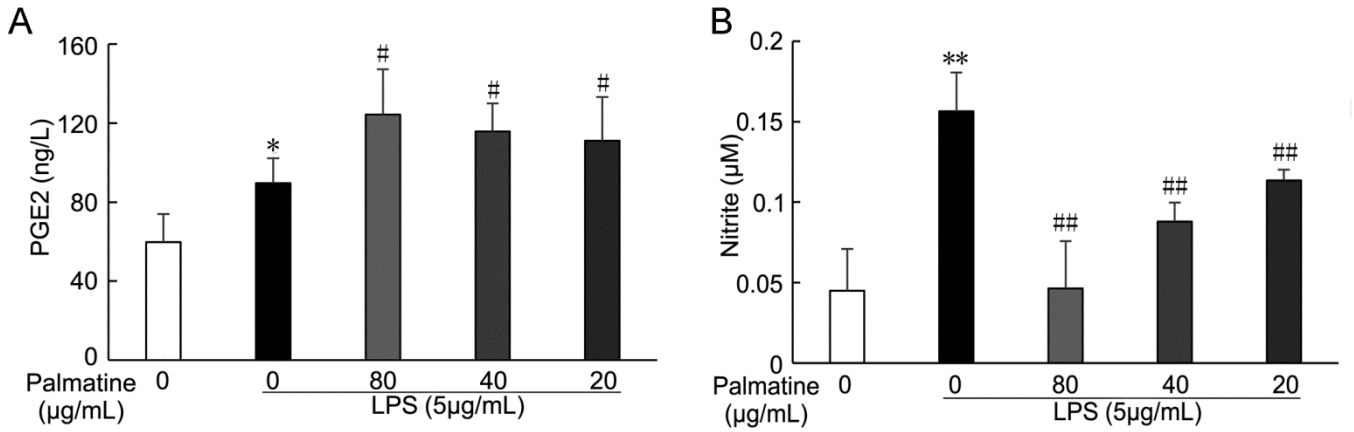

Fig.4. Modulatory effects of the palmatine (20, 40 and 80 μg/mL) on the level of NO (B) and PGE_2 (A) in LPS-stimulated gEECs. The cells were treated with the palmatine for 8 h after LPS administration. The control group treated without palmatine and LPS. Statistical differences between groups represented with (**) P< 0.01 vs. control group, (##) P< 0.01 vs. LPS group and (#) P< 0.05 vs. LPS group, values are expressed as mean ± SD (n = 3).

3.4. Palmatine inhibited MMP-2 and MMP-9 in LPS-stimulated EECs

The result of Fig.5 indicated that LPS significantly promoted the production of MMP-2 and MMP-9 compared with the control group ($P < 0.05$) and palamine treatment significantly inhibit the secretion of MMP-2 and MMP-9.

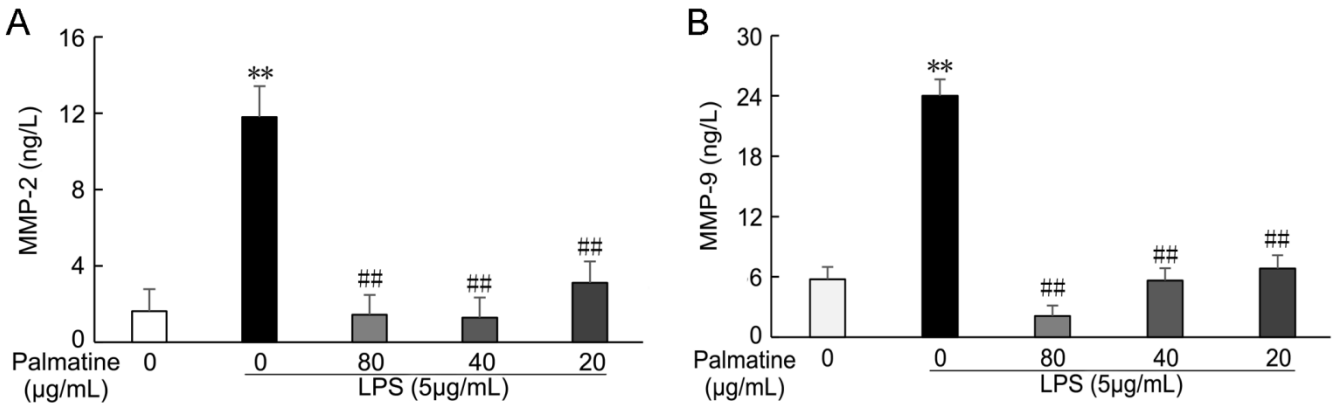

Fig.5. Modulatory effects of the palmatine (20, 40 and 80 μg/mL) on the level of MMP-9(B) and MMP-2(A) in LPS-stimulated gEECs. The cells were treated with the palmatine for 8 h after LPS administration. The control group treated without palmatine and LPS. Statistical differences between groups represented with (**) P< 0.01 vs. control group, (##) P< 0.01 vs. LPS group and (#) P< 0.05 vs. LPS group, values are expressed as mean ± SD (n = 3).

3.5. Effects of Palmatine on gene expression in LPS-stimulated EECs

As shown in Fig.6 LPS significantly up-regulated gene expression of IL-1β, TLR4, CD14

and TICAM compared with control group ($P < 0.05$). When LPS stimulated-gEECs were exposured to palamine, the gene expressions of IL-1β, TLR4, CD14 and TICAM were down-regulated intensively. Noted that there was no distinct changes of MyD88 in either LPS group or palamine treatment groups ($P > 0.05$).

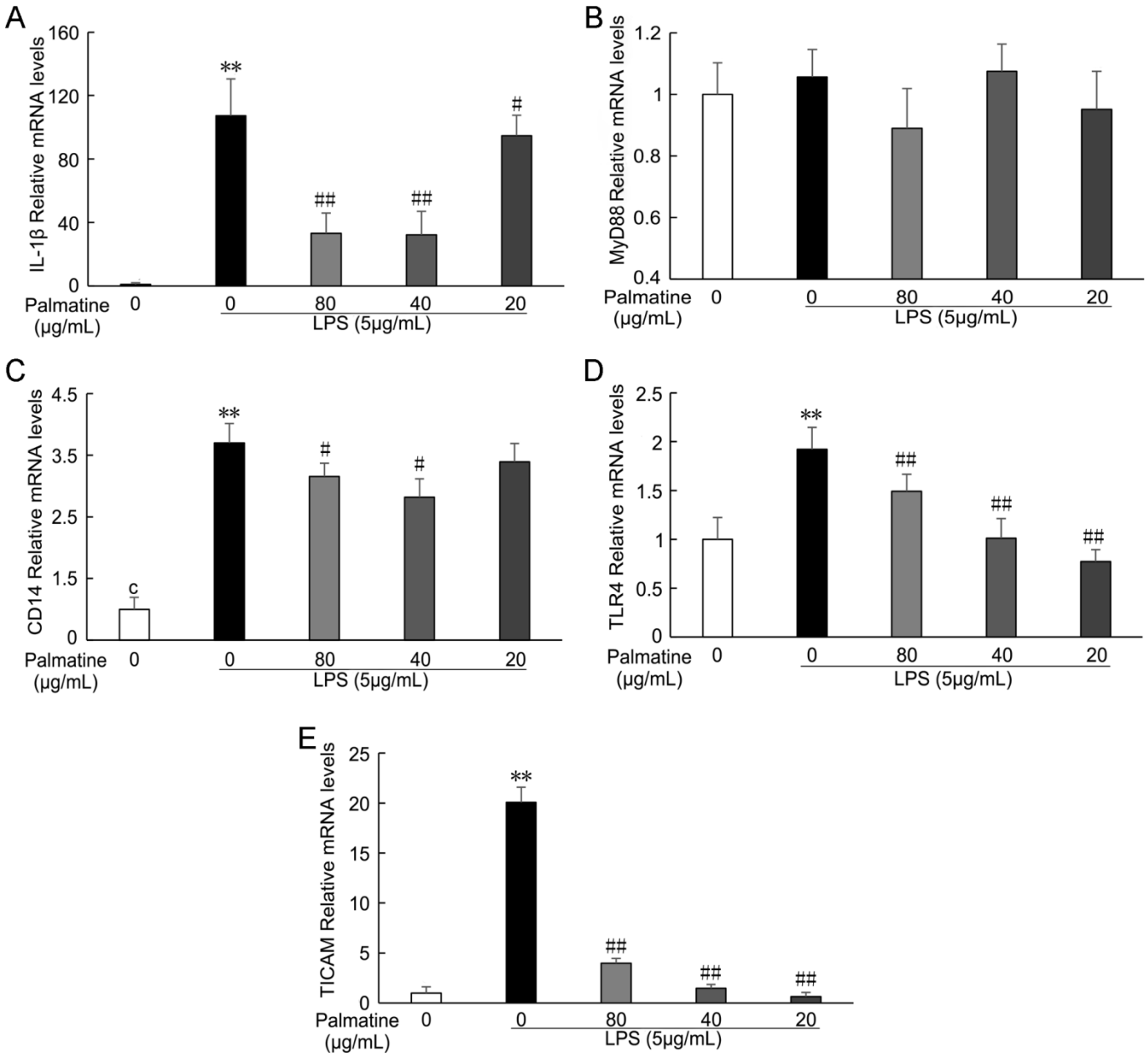

Fig.6. Modulatory effects of the palmatine (20, 40 and 80 μg/mL) on the gene expression in the gEECs induced by LPS, IL-1 β (A), MyD88(B), CD14 (C), TLR4 (D), TICAM (E). The cells were treated with the palmatine for 8 h after LPS administration. The control group treated without palmatine and LPS. Statistical differences between groups represented with (**) P< 0.01 vs. control group, (##) P< 0.01 vs. LPS group and (#) P< 0.05 vs. LPS group, values are expressed as mean ± SD (n = 3).

3.6. Palmatine inhibited protein and mRNA expression of NF-κB

The Fig.7 demonstrated that the relative mRNA level and protein expression of NF-κBp65 increased markedly with the stimulation of LPS compared with the control group ($P < 0.05$). Palmatine at 40 and 20 μg/mL did not affect the mRNA expression of NF-κBp65. But palmatine at 80 μg/mL significantly inhibited the mRNA level of NF-κBp65 with the LPS group ($P < 0.05$). It showed that when palmatine reached a certain concentration it will significantly inhibit the gene expression of NF-κBp65. The relative protein level of NF-κBp65 was calculated as NF-κBp65/ β-actin. The result declined that palmatine inhibit the protein expression of phosphorylated p65. Palmatin at 80 μg/mL exhibited the most obvious inhibitory effect. In general, palmatine suppressed the gene expression and the phosphorylation of NF-κBp65.

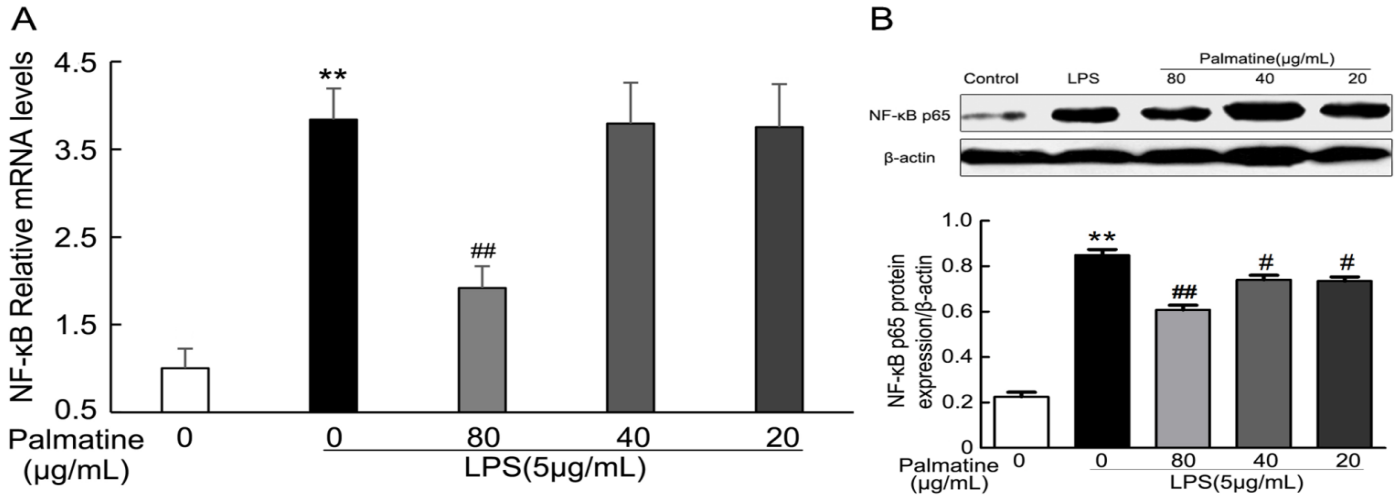

Fig.7. Modulatory effects of the palmatine (20, 40 and 80 μg/mL) on the NF- κ B. (A)NF- κ B mRNA expression and (B) NF- κ Bp65 protein levels in LPS-stimulated gEECs. The cells were treated with the palmatine for 8 h after LPS stimulation. The control group treated without palmatine and LPS. Statistical differences between groups represented with (**) P< 0.01 vs. control group, (##) P< 0.01 vs. LPS group and (#) P< 0.05 vs. LPS group, values are expressed as mean ± SD (n = 3).

4. Discussion

Endometritis is one of the top problems veterinarians face in clinical practice. It has been reported that 14%～53% of cows as well as 10%～20% of mares will develop into some types of endometritis [23, 24]. In recent years, with the multiplication of drug resistant bacteria and the

excessive residues of antibiotics, the study of Chinese herbal medicine for the treatment of endometritis became a research hotspot. Palmatine, an important traditional medicinal compound, has various biological activities usually used to treat jaundice, hypertension, dysentery, inflammation, and liver-related diseases [17]. However there are few reports about the anti-inflammatory effects of palmatine on endometritis in veterinary. EECs have been used as an i*n vitro* model system in endometrial disease research for many years. It was used to study the first line of defense against invading organisms, known as innate immunity [25]. In the present study, we used an LPS-induced gEECs inflammartion model to evaluate the anti-inflammatory activity of palmatine. We found that 5 μg/mL LPS could successfully induce inflammation after its treatment for 12 h in gEECs (data not shown). The results of Fig.3 showed that the level of pro-inflammatory factors TNF-α, IL-1β and IL-6 increased significantly in the LPS group compared with the control group ($p< 0.05$). These results indicated that LPS-stimulated gEECs inflammation model was successful. And it can be used to further experiment.

TNF-α and IL-6 are important pro-inflammatory cytokines [26]. Previous studies reported that animals with endometritis have higher inflammatory cytokines concentrations in uterine flush than healthy ones [27]. TNF-α is able to activate other inflammatory cytokines like IL-1β and induce the expression of adhesion molecules [28]. IL-6 is responsible for the coordination of the acute phase response and plays an important role in the local inflammatory reaction by amplifying leukocyte accumulation [29]. IL-1β mobilizes neutrophils followed by phagocytosis of invading pathogens within the uterine lumen [30]. NO, an inflammatory mediator, its high concentration can cause vasodilatation and increase inflammation-based permeability and amount of mononuclear cells at the site of inflammation [31]. In present study, palmatine had showed an anti-inflammatory effect by significantly inhibiting the secretion of

the above pro-inflammatory factors in a dose-dependent manner (Fig.3, Fig.4).

IL-10 acts as a potent anti-inflammatory cytokine that functions to resolve inflammatory response by limiting the expression of pro-inflammatory cytokines and chemokines. It protects the host from excessive inflammation [32, 33]. As reported previously, endometritis had a concomitant increase of anti-inflammatory cytokines, such as IL-10, it may be desirable to administer immune stimulatory preparations [34]. In our study, palmatine at 80 μg/mL significantly decreased the level of IL-10, but its lower concentrations (40 and 20 μg/mL) significantly raised IL-10 production (Fig.3). The reason of this was that anti-inflammatory effect of the palmatine at 80 μg/mL had already reached a certain level, even below normal level (vs control group $p< 0.05$). Hardly there were any inflammatory effects in model, so the secretion of anti-inflammatory cytokine IL-10 was inhibited. While, after the treatment of palmatine at 40 and 20 μg/mL, inflammation still existed in LPS-induced EECs. So the secretion of IL-10 was up-regulated by palmatine to inhibit pro-inflammatory cytokines.

In previous research, the synthesis of PGE_2 was stimulated by various factors involved in inflammation. It modulates the inflammatory response, including vasodilatation and the production of various cytokines. Previous studies reported the higher concentration of PGE_2 in blood plasma or uterine fluid during endometritis [35, 36]. Our results demonstrated that palmatine promoted the release of PGE_2. It suggested that palmatine may enhance PGE_2 to inhibit inflammation as PGE_2 exerts both pro- and anti-inflammatory effects combining with changes of the cytokines [37, 38].

Matrix metalloproteinase (MMP) is a key enzyme in collagen metabolism. It can occur in fibroblasts and epithelial cells as an important regulator of inflammation [39]. MMP-2 and MMP-9 are important enzymes in the degradation of the extracellular matrix (EMC) and with a destructive effect on the integrity of the basement membrane [40]. When endometritis

occurred early, MMPs produced massively. Inhibition of its production would protect endometrium from structure degradation (Fig.5).

Previous studies have indicated that LPS is recognized by TLR4, which leads to the activation of two different signaling pathways: MyD88-dependent and TRIF-dependent. TLR4- and MyD88-dependent signaling pathways are essential for the response to LPS by the epithelial and stromal cells in the bovine endometrium and activated NF-κB up-regulates the transcription of pro-inflammatory genes such as TNF-α, IL-6 [41-43]. In MyD88 knock-out mice macrophage, TRIF is recruited to TLR4 and activates an alternative pathway that triggers the activation of NF-κB, MAPKs, and IRF3. These signaling cascades lead to the production of pro-inflammatory cytokines, type I interferons (IFNs), chemokines, and antimicrobial peptides to remove the invading pathogens [44, 45]. It was reported that the TLR4 mRNA and protein levels significantly increased in LPS-induced mammary gland tissues [46]. According to these theories, we detected the genes expression of TLR4 and CD14. Our results revealed that the gene expression of TLR4 and CD14 increased significantly in LPS-induced gEECs inflammartion model. That was in agreement with previous studies. We examined the mRNA expression of TRIF (TICAM) and found that LPS significantly up-regulated the transcription of TRIF. Palmatine treatment down-regulated the gene levels of TLR4, CD14 and TRIF in TLR4 signaling pathways, but it did not affect MyD88 expression. It is well known that IL-1β, TNF-α and IL-6 expression is modulated by the NF-κB and MAPK pathways, which are signaling pathways down-stream of TRIF-dependent TLR4. Activated NF-κB subunits translocate to the nucleus and bind to the promoters of target genes to activate their transcription [42, 47]. To further assess the mechanism of Palmatine anti-inflammatory effect, we tested the gene expression of NF-κB and the protein expression of activated NF-κB. The results showed that Palmatine down-regulated the expression of NF-κB and suppressed the action of

NF-κB.

In summary, Palmatine possesses anti-inflammatory bioactivities in LPS-induced gEECs inflammation model. It decreases the production of pro-inflammatory factors, such as TNF-α, IL-1β, IL-6 and NO and increases the production of anti-inflammatory IL-10 and pro-resolution mediator and PGE_2. In addition, palmatine protect gEECs from inflammatory injury by inhibiting the production of MMP2. As summarized in Fig.8, our results suggest that the anti-inflammatory mechanism of palmatine was related to the inhibition of TRIF-dependent and NF-κB signaling pathways at the gene level.

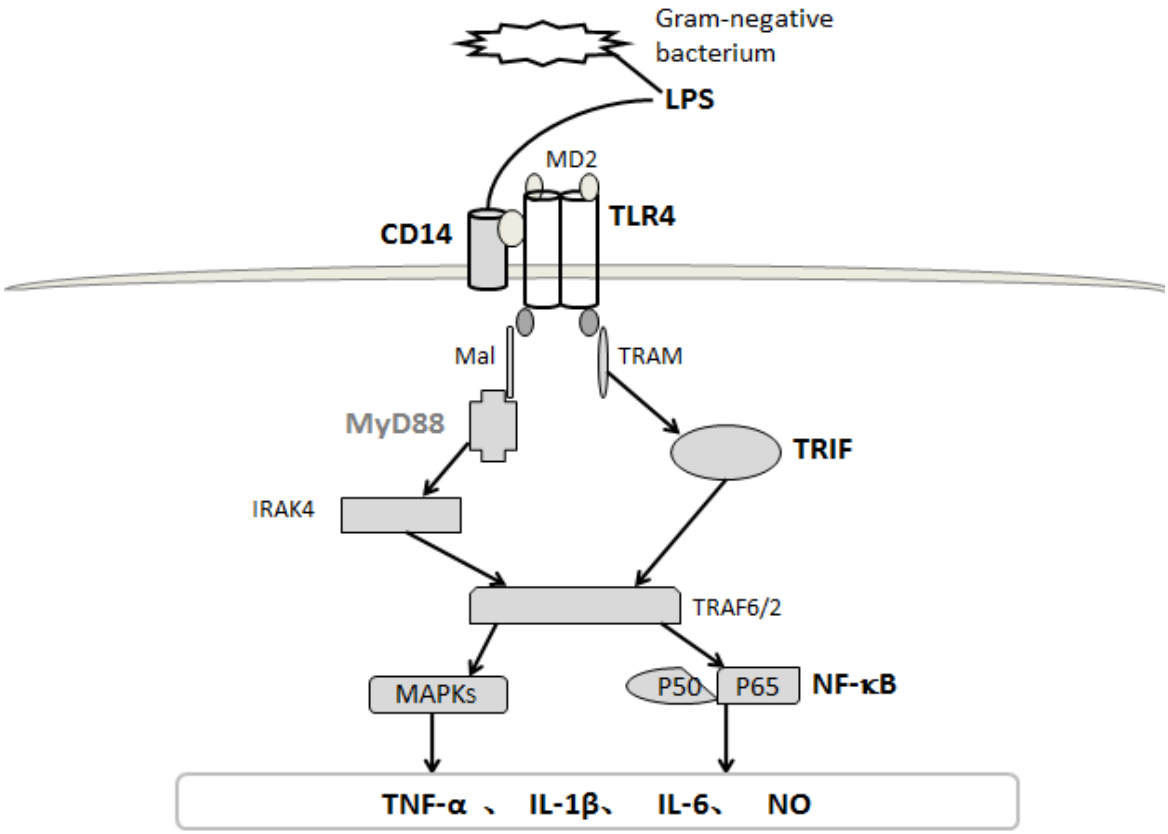

Fig.8. The signal pathways of the anti-inflammatory mechanism of palmatine. LPS significantly increased the gene expressions of TLR4, CD14, TRIF and NF- κ B ($P < 0.05$). Palmatine can significantly decrease the levels of them. Otherwise the level of MyD88 had no changes ($P < 0.05$).

5. Acknowledgments

This work was supported by Lanzhou institute of husbandry and pharmaceutical sciences of CAAS and guided by Zuo-ting Yan research and doctor Shi-dong Zhang. This dissertation

was funded jointly by three projects, which are the project of national science and technology supporting plan of china (2012BAD12B03), and the project of national science and technology supporting plan of Gansu province (1506RJYA146) and the Agricultural Science and Technology Innovation Program (CAAS-ASTIP-2014-LIHPS-03).

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