



## Punicalagin protects bovine endometrial epithelial cells against lipopolysaccharide-induced inflammatory injury<sup>\*</sup>

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**Abstract:** Objective: Bovine endometritis is one of the most common reproductive disorders in cattle. The aim of this study was to investigate the anti-inflammation potential of punicalagin in lipopolysaccharide (LPS)-induced bovine endometrial epithelial cells (bEECs) and to uncover the underlying mechanisms. Methods: bEECs were stimulated with different concentrations (1, 10, 30, 50, and 100 µg/ml) of LPS for 3, 6, 9, 12, and 18 h. MTT assay was used to assess cell viability and to identify the conditions for inflammatory injury and effective concentrations of punicalagin. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to assess gene expression of pro-inflammatory cytokines. Western blotting was used to assess levels of inflammation-related proteins. Results: Treatment of bEECs with 30 µg/ml LPS for 12 h induced cell injury and reduced cell viability. Punicalagin (5, 10, or 20 µg/ml) pretreatment significantly decreased LPS-induced productions of interleukin (IL)-1 $\beta$ , IL-6, IL-8, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in bEECs. Molecular research showed that punicalagin inhibited the activation of the upstream mediator nuclear factor- $\kappa$ B (NF- $\kappa$ B) by suppressing the production of inhibitor  $\kappa$ B $\alpha$  (I $\kappa$ B $\alpha$ ) and phosphorylation of p65. Results also indicated that punicalagin can suppress the phosphorylation of mitogen-activated protein kinases (MAPKs) including p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK). Conclusions: Punicalagin may attenuate LPS-induced inflammatory injury and provide a potential option for the treatment of dairy cows with *Escherichia coli* endometritis.

**Key words:** Bovine endometrial epithelial cell; Cytokine; Inflammatory injury; Punicalagin  
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### 1 Introduction


Endometritis is one of the most common reproductive disorders caused by bacteria in cattle throughout the world. It damages the endometrium, delaying the onset of the ovarian cycle activity after calving, extending luteal phases, reducing fertility,

and causing significant economic losses (Sheldon *et al.*, 2006; Azawi, 2008; Kasimanickam *et al.*, 2013). Therefore, methods are needed to eradicate the causative bacteria earlier and control inflammation. Although antibiotics have been effective, increasing drug resistance and concerns about food safety limit their use (Malinowski *et al.*, 2011; Zhao *et al.*, 2014; Mackeen *et al.*, 2015; Ward and Duff, 2016).

*Escherichia coli* and other Gram-negative bacteria are the major pathogens associated with endometritis (Janowski *et al.*, 2013; Sens and Heuwieser, 2013; Brodzki *et al.*, 2014; Wagener *et al.*, 2014). Lipopolysaccharides (LPSs) are the major structural components of the cell wall of Gram-negative bacteria.

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LPS can be transferred to cluster of differentiation 14 (CD14) by LPS-binding protein (LBP) and is recognized by Toll-like receptor 4 (TLR4) on the cell surface (Shimada *et al.*, 2005; Khan *et al.*, 2009; Wu *et al.*, 2016). The interaction between LPS and TLR4 results in the activation of intracellular signaling through myeloid differentiation factor 88 (MyD88) and TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF) pathways, leading to the activation of major mitogen-activated protein kinases (MAPKs) and translocation of nuclear transcription factor- $\kappa$ B (NF- $\kappa$ B) (Sheldon and Roberts, 2010; Kawai and Akira, 2011; Huang *et al.*, 2016). NF- $\kappa$ B regulates the expression of cytokines, including interleukin (IL)-1 $\beta$ , IL-6, IL-8, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), which are essential mediators of the inflammation response (Wang *et al.*, 2012; Liu *et al.*, 2014; Huang *et al.*, 2016).

The endometrium is the first line of defense against microbial invasion, and endometrial epithelial cells are thought to play a key role in local innate immunity (Herath *et al.*, 2006; Soboll *et al.*, 2006; Turner *et al.*, 2014). Endometrial epithelial cells line the uterine mucosal surface, forming a physical barrier to protect the host from pathogen invasion (Wira *et al.*, 2005). Endometrial epithelial cells in the bovine uterus were found to recognize and respond to pathogens via surface receptors such as TLRs (Chapwanya *et al.*, 2013; Turner *et al.*, 2014).

Punicalagin, a hydrolysable tannin of pomegranate juice, is a powerful nutrient that promotes overall health (Yaidikar *et al.*, 2014). Punicalagin exhibits multiple biological effects, including antioxidant, antiproliferative, antiviral, and antimicrobial activities (Taguri *et al.*, 2004; Aqil *et al.*, 2012; Yang *et al.*, 2012; Xu *et al.*, 2016). Moreover, it has shown anti-inflammatory properties both in vitro and in vivo (Jean-Gilles *et al.*, 2013; Olajide *et al.*, 2014; Xu *et al.*, 2014). However, the effects of punicalagin on endometritis have not been investigated. This study was therefore designed to investigate whether punicalagin could protect against LPS-induced inflammatory injury in bovine endometrial epithelial cells (bEECs) and to clarify its possible mechanisms of action.

## 2 Materials and methods

### 2.1 Reagents

Punicalagin (>98% high-performance liquid chromatography (HPLC) purity) was purchased from

Tauto Biotech (Shanghai, China). LPS (*E. coli* O55:B5), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), trypsin, collagenase II, bovine serum albumin (BSA), and DNase I were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture (DMEM/F12), fetal bovine serum (FBS), antibiotic, and TRIzol reagent were purchased from Gibco (Grand Island, NY, USA). A bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL, USA). Antibodies against  $\beta$ -actin, inhibitor  $\kappa$ B $\alpha$  (I $\kappa$ B $\alpha$ ), phosphorylated p65 (p-p65), phosphorylated p38 (p-p38), phosphorylated c-Jun N-terminal kinase (p-JNK), and phosphorylated extracellular signal-regulated kinase (p-ERK) were purchased from Cell Signaling Technology (Danvers, MA, USA). Goat anti-mouse antibody was purchased from Li-CDR Odyssey (Lincoln, NE, USA).

### 2.2 Cell culture and treatment

Uteruses from five non-pregnant Holstein cows were obtained from a local abattoir immediately after slaughter and kept on ice until further processing in the laboratory. The animals had no evidence of genital disease based on visual inspection and the uteruses were obtained in accordance with protocols approved by the local Institutional Animal Care and Use Committee. Primary bEECs were isolated from the endometrium as described previously (Chapwanya *et al.*, 2013). In brief, the endometrium was sliced into small pieces measuring about 3–5 mm<sup>3</sup> and digested with sterile filtered digestive solution (50 mg trypsin, 50 mg collagenase II, 100 mg BSA, and 10 mg DNase I) for 1 h in a gently agitating water bath at 37 °C. Cells were resuspended in DMEM/F12 containing 10% FBS and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin) at 37 °C in a humidified incubator with 5% CO<sub>2</sub>.

### 2.3 Cell viability assay

The bEECs were seeded in 96-well plates at 1 $\times$ 10<sup>4</sup> cells per well and allowed to grow to 90% confluence in complete medium. After 24 h, cells were washed twice with phosphate-buffered saline (PBS) and incubated with serum-free medium for 1 h at 37 °C in 5% CO<sub>2</sub>. Cells were then treated with different concentrations of LPS (0, 1, 10, 30, 50, and 100  $\mu$ g/ml) for 3, 6, 9, 12, or 18 h in the incubator. Cell viability was measured by the MTT assay. To

examine the cytotoxicity of punicalagin in bEECs, cells were treated with different concentrations of punicalagin (5, 10, 20, 25, 30, and 50  $\mu\text{g/ml}$ ) for 24 h. The medium was then removed and DMEM/F12 containing 10% MTT was added to each well. Formazan complex was dissolved in 100  $\mu\text{l}$  dimethyl sulfoxide (DMSO) after 4 h of incubation. The optical density was measured using a microplate reader (Bio-Rad, USA) at 490 nm.

#### 2.4 Quantitative real-time PCR analysis

To detect the effects of punicalagin on gene expression in LPS-stimulated cells, bEECs were pre-incubated in six-well plates ( $1 \times 10^6$  cells/well) and pretreated with punicalagin (5, 10, and 20  $\mu\text{g/ml}$ ) for 2 h prior to LPS (30  $\mu\text{g/ml}$ ) treatment in an incubator at 37 °C and 5% CO<sub>2</sub> for 3, 6, 9, or 12 h. Total RNA was isolated using a phenol and guanidine isothiocyanate-based TRIzol reagent according to the manufacturer's instructions. The concentration and integrity of total RNA were measured at a 260/280 nm ratio. Quantitative polymerase chain reaction (PCR) analysis was performed using the DNA Engine Mx3000P<sup>®</sup> (Agilent, Santa Clara, CA, USA) fluorescence detection system against a double-stranded DNA-specific fluorescent dye (Stratagene, La Jolla, CA, USA) according to optimized PCR protocols.  *$\beta$ -Actin* was amplified in parallel with the target genes and used as a normalization control. The cycling conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 60 s, and 72 °C for 60 s. Expression levels were determined using the relative threshold cycle (Ct) method as described by the manufacturer (Stratagene). The PCR reaction system (25  $\mu\text{l}$  in total) contained 12.5  $\mu\text{l}$  of SYBR Green PCR

mix, 0.375  $\mu\text{l}$  of reference dye, 1  $\mu\text{l}$  of each primer (both, 10  $\mu\text{mol/L}$ ), 1  $\mu\text{l}$  of complementary DNA (cDNA) template, and 9.125  $\mu\text{l}$  of diethyl phosphorocyanidate (DEPC)-treated water. Table 1 lists the gene-specific oligonucleotide primers used for real-time PCR (RT-PCR).

#### 2.5 Western blot analysis

The bEECs cultured in tissue culture flasks for 24 h were pretreated with punicalagin for 2 h prior to treatment with LPS (30  $\mu\text{g/ml}$ ) for 15, 45, or 90 min in an incubator at 37 °C and 5% CO<sub>2</sub>. Cells were then harvested on ice, washed twice using ice-cold PBS, and suspended in 500  $\mu\text{l}$  lysis buffer supplemented with protease inhibitor. After incubating on ice for 30 min, cell extracts were subjected to centrifugation (12000g) at 4 °C for 15 min and proteins were quantified using a BCA protein assay kit. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to nitrocellulose membranes (Pierce Biotechnology Inc.), which were then hybridized with the specific antibodies.  *$\beta$ -Actin* was used to correct for protein loading. Densitometric values of immunoblot signals were obtained from three separate experiments using Image J software (National Institutes of Health, USA).

#### 2.6 Statistical analysis

The results are expressed as mean  $\pm$  the standard error of the mean (SEM) and differences between mean values of normally distributed data were assessed by one-way analysis of variance (ANOVA) multiple comparisons. A *P*-value of 0.05 or less was regarded as significant.

Table 1 Primers for RT-PCR

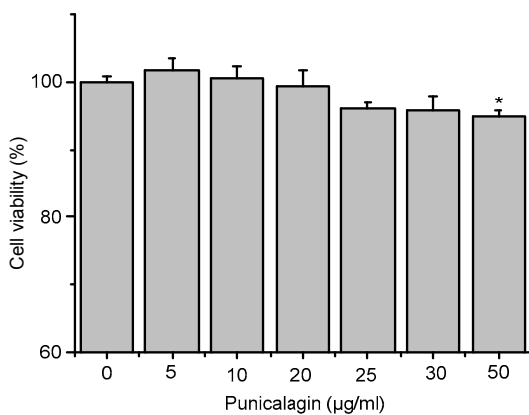
Gene	Accession number <sup>1</sup>	Primer sequence (5'→3')	Product (bp)
<i><math>\beta</math>-actin</i>	NM_173979.3	F: CAGAAGGACTCGTACGTGGG R: TTGGCCTTGGGGTTCAGGG	199
<i>TNF-<math>\alpha</math></i>	NM_173966.3	F: CTTTATTGCCAGGTTTCTG R: CAGGTGTTGGATGCAGCTCT	141
<i>IL-1<math>\beta</math></i>	NM_174093.1	F: ATGACTTCTGCTTCCCTACCC R: GCTGCTTTCACACTCATCATTC	179
<i>IL-6</i>	NM_173923.2	F: GCAFFTATTTGTGAAGAGAGCTG R: CACAGAACATGAGGCACTGAA	148
<i>IL-8</i>	NM_173925.2	F: ACGGGCTTTACCTCATCTACTC R: GCTCTTGATGGCAGACAGG	140

<sup>1</sup> Primers were designed from the sequences published in the GenBank database under the indicated accession numbers. F: forward primer; R: reverse primer

### 3 Results

#### 3.1 Effect of punicalagin on cell viability

We first examined the cytotoxicity of different concentrations of punicalagin (5, 10, 20, 25, 30, and 50  $\mu\text{g/ml}$ ) on bEECs by evaluating cell viability using the MTT assay. The results showed that punicalagin in concentrations from 0 to 30  $\mu\text{g/ml}$  had no cytotoxic effect on bEECs (Fig. 1), suggesting that the inhibitory effect of punicalagin on LPS-induced inflammation was not a result of cytotoxicity caused by a reduction in cell viability.

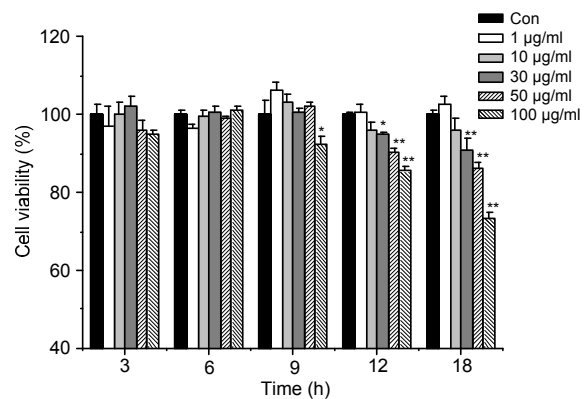


**Fig. 1 Punicalagin cytotoxicity in bEECs**

Cells were treated with different concentrations (5, 10, 20, 25, 30, and 50  $\mu\text{g/ml}$ ) of punicalagin for 24 h. Cell viability was evaluated by MTT assay. Data represent the mean  $\pm$  SEM of three independent experiments. \*  $P < 0.05$  vs. the control group

#### 3.2 Inhibition of LPS-induced pro-inflammatory cytokine production by punicalagin

To determine the appropriate LPS concentration and time for stimulation, cell viability was tested after LPS treatment with different concentrations (0, 1, 10, 30, 50, and 100  $\mu\text{g/ml}$ ) for 3, 6, 9, 12, and 18 h. Cell viability was not altered by the treatment with 1 and 10  $\mu\text{g/ml}$  of LPS before 12 h (Fig. 2). However, stimulation of cells with 30  $\mu\text{g/ml}$  LPS for 12 h significantly reduced cell vitality ( $P < 0.05$ ), and compared with the control group (Figs. 3a–3c), the treated cells showed obvious floating, rounding, and nuclear pycnosis (Figs. 3d–3f). The effects of 50 and 100  $\mu\text{g/ml}$  LPS on cell viability after 12 h were more pronounced ( $P < 0.01$  each). Therefore, we selected cell stimulation with 30  $\mu\text{g/ml}$  LPS for 12 h for our experimental analyses.



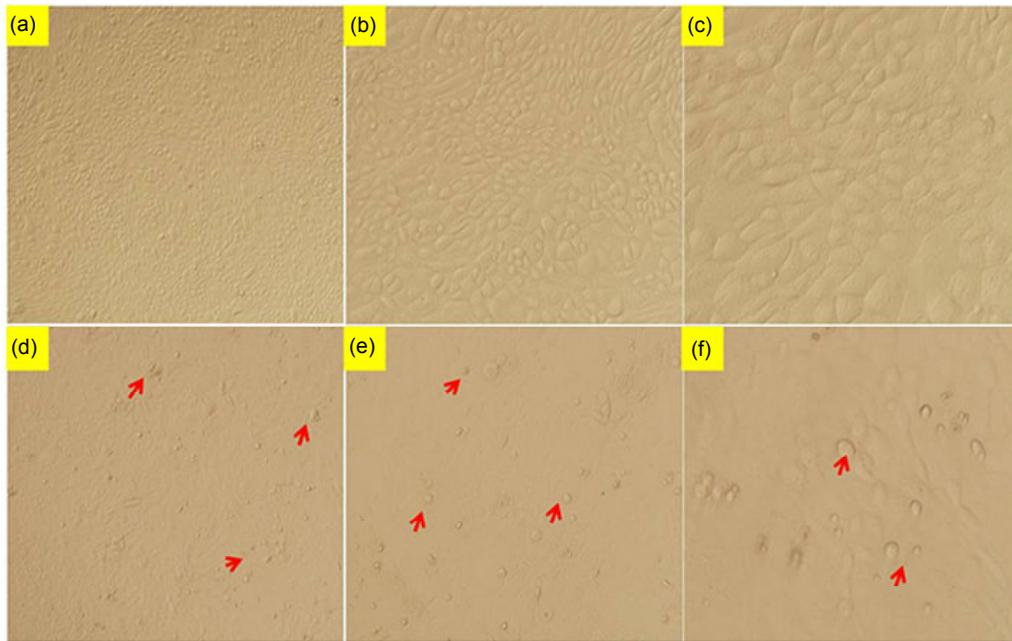
**Fig. 2 Cell viability of bEECs induced by LPS**

Cells were incubated with different concentrations (1, 10, 30, 50, and 100  $\mu\text{g/ml}$ ) of LPS for 3, 6, 9, 12, and 18 h. Cell viability was significantly reduced by 30  $\mu\text{g/ml}$  of LPS at 12 h (\*  $P < 0.05$ ). Data represent the mean  $\pm$  SEM of three independent experiments. \*  $P < 0.05$ , \*\*  $P < 0.01$  vs. the control (Con) group

To determine the effect of punicalagin on LPS-induced pro-inflammatory cytokine expression, total RNA was extracted and examined by RT-PCR. The expression levels of IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$  induced by LPS were significantly ( $P < 0.001$ ) upregulated at indicated time points (Fig. 4). This effect was markedly inhibited by punicalagin in a dose-dependent manner, confirming that punicalagin had anti-inflammatory effects.

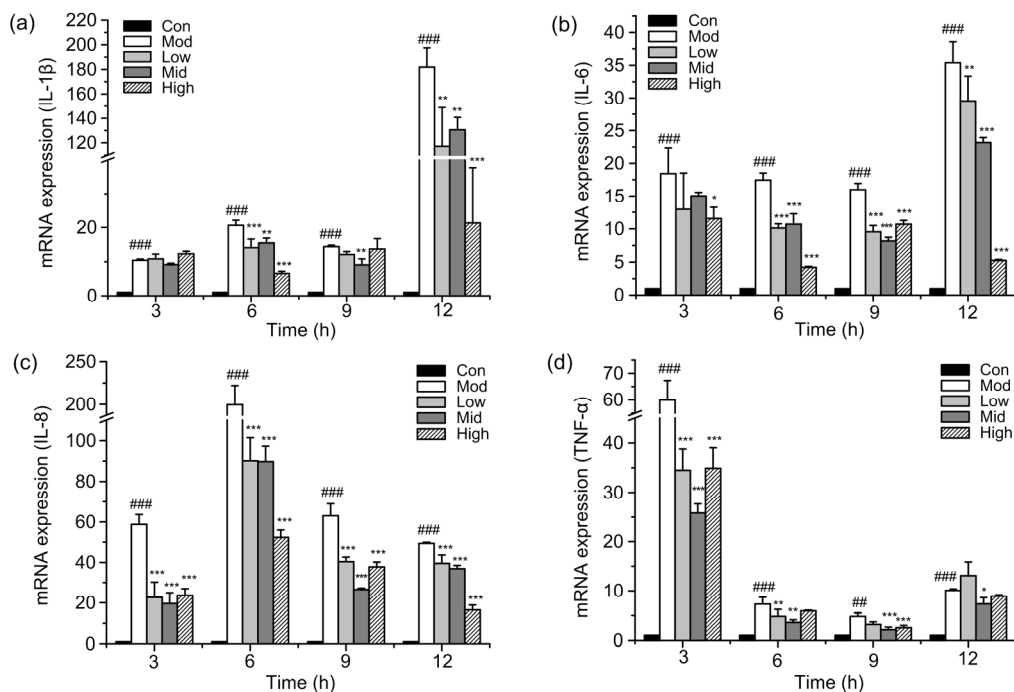
#### 3.3 Inhibition of LPS-induced NF- $\kappa$ B activation by punicalagin

NF- $\kappa$ B is an important transcription factor that induces cytokine mRNA after stimulation by LPS. Therefore, critical proteins involved in NF- $\kappa$ B signaling pathway were examined by Western blotting. The results showed an almost complete degradation of I $\kappa$ B $\alpha$  and a significant increase in phosphorylation of p65 after cells were treated with LPS for 15 min (Figs. 5a and 5b), indicating an increase in NF- $\kappa$ B activity. The degradation of I $\kappa$ B $\alpha$  and phosphorylation of p65 induced by LPS were partially inhibited by punicalagin, which indicated a weakening activity of NF- $\kappa$ B. After 45 min, the degradation of I $\kappa$ B $\alpha$  and phosphorylation of p65 had decreased in all groups. These results show that NF- $\kappa$ B activity in bEECs induced by LPS was significantly inhibited by punicalagin.



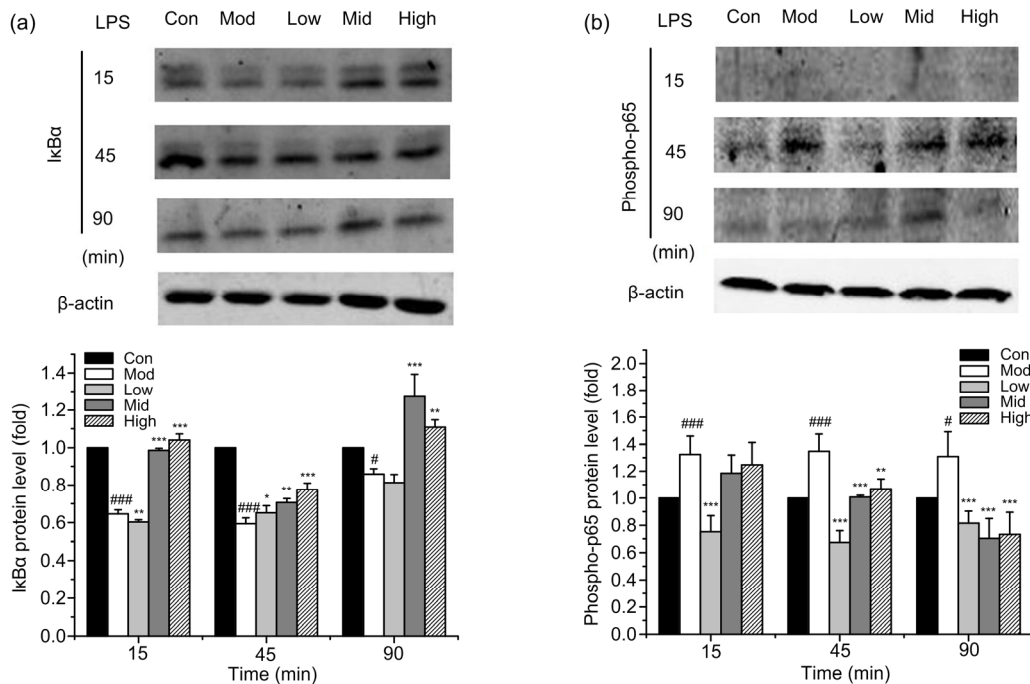
**Fig. 3 Morphology of bEECs induced by LPS**

Cells were incubated with or without 30  $\mu\text{g/ml}$  LPS for 12 h and cell morphology was observed using an inverted microscope. (a–c) Control group: the distribution of bEECs was compact and regular. (d–f) Model group: bEECs exposed to 30  $\mu\text{g/ml}$  LPS for 12 h became enlarged, lost their cuboidal shape, and showed disrupted cell-cell contacts (arrowheads). (a, d) 4 $\times$  microscope; (b, e) 10 $\times$  microscope; (c, f) 20 $\times$  microscope



**Fig. 4 Effect of punicalagin on LPS-induced pro-inflammatory cytokine mRNA expression**

Cells were pretreated with punicalagin (5, 10, and 20  $\mu\text{g/ml}$ ) for 2 h and exposed to 30  $\mu\text{g/ml}$  LPS for 3, 6, 9, and 12 h. The levels of IL-1 $\beta$  (a), IL-6 (b), IL-8 (c), and TNF- $\alpha$  (d) mRNAs were quantified using RT-PCR analysis. Data represent the mean $\pm$ SEM of three independent experiments. #  $P < 0.01$ , ##  $P < 0.001$  vs. the control (Con) group. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  vs. the model (Mod) group. Con: control; Mod: treated with LPS (30  $\mu\text{g/ml}$ ) only; Low: punicalagin (5  $\mu\text{g/ml}$ )+LPS (30  $\mu\text{g/ml}$ ); Mid: punicalagin (10  $\mu\text{g/ml}$ )+LPS (30  $\mu\text{g/ml}$ ); High: punicalagin (20  $\mu\text{g/ml}$ )+LPS (30  $\mu\text{g/ml}$ )



**Fig. 5 Effect of punicalagin on LPS-induced NF-κB activation**

Cells were pretreated with punicalagin (5, 10, and 20  $\mu\text{g/ml}$ ) for 2 h, exposed to 30  $\mu\text{g/ml}$  LPS for 15, 45, and 90 min, and analyzed by Western blotting. I $\kappa$ B $\alpha$  (a) and phosphorylated p65 (b) were analyzed using anti-I $\kappa$ B $\alpha$  and phosphor-specific anti-p65 antibodies. Data represent the mean $\pm$ SEM of three independent experiments. <sup>#</sup> $P$ <0.05, <sup>###</sup> $P$ <0.001 vs. the control (Con) group. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 vs. the model (Mod) group. Con: control; Mod: treated with LPS (30  $\mu\text{g/ml}$ ) only; Low: punicalagin (5  $\mu\text{g/ml}$ )+LPS (30  $\mu\text{g/ml}$ ); Mid: punicalagin (10  $\mu\text{g/ml}$ )+LPS (30  $\mu\text{g/ml}$ ); High: punicalagin (20  $\mu\text{g/ml}$ )+LPS (30  $\mu\text{g/ml}$ )

### 3.4 Inhibition of LPS-induced MAPK activation by punicalagin

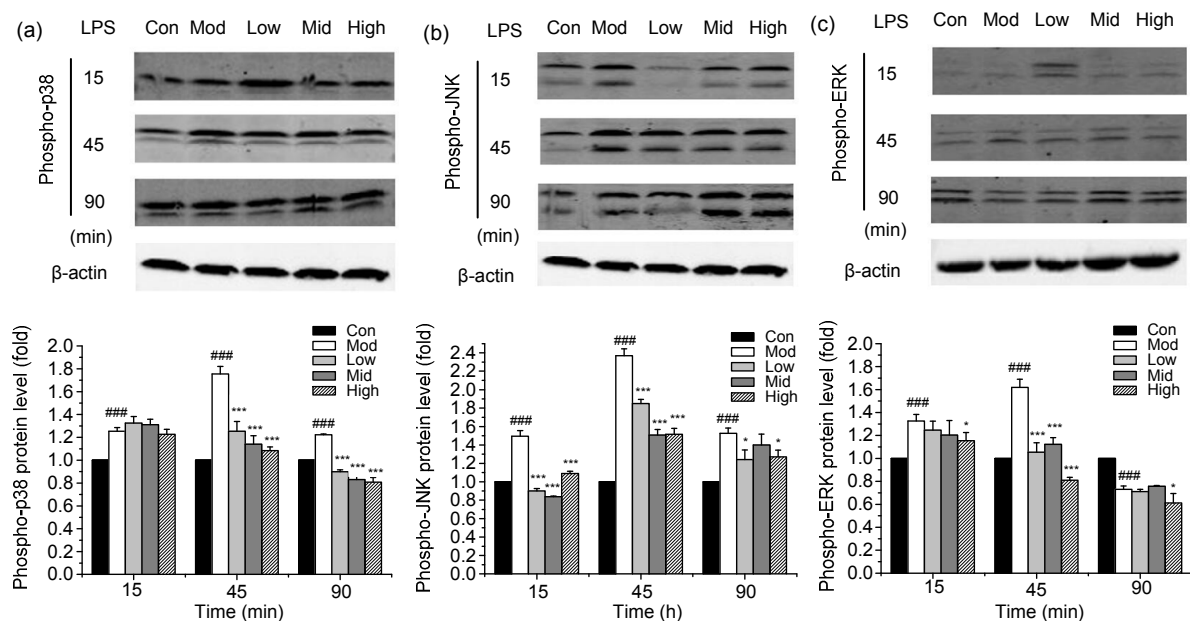
To further expound the mechanism of the inhibitory effect on LPS-induced pro-inflammatory cytokine expression by punicalagin, we then investigated the effect of punicalagin on LPS-induced activation via the MAPK signaling pathway by assessing the phosphorylation of p38, JNK, and ERK (Fig. 6). Treatment of bEECs with LPS significantly ( $P$ <0.001) increased the activation of MAPKs by strengthening the phosphorylation of p38, JNK, and ERK. However, the phosphorylation levels were attenuated to some degree in punicalagin-pretreated cells compared with LPS-treated cells.

## 4 Discussion

LPS, a cell wall component of Gram-negative bacteria, is a well-known inducer of inflammation, and exposure of cells to LPS has been shown to promote the release of pro-inflammatory cytokines

(Lim *et al.*, 2012; Fu *et al.*, 2013; Wang *et al.*, 2013), making LPS a good stimulant of inflammation. In many previous reports, the concentrations of LPS used in vitro were less than 1  $\mu\text{g/ml}$  (Choi *et al.*, 2007; Wang *et al.*, 2012; Qi *et al.*, 2016). However, in our study, the treatment with LPS up to 30  $\mu\text{g/ml}$  for 3, 6, or 9 h did not inhibit cell viability. This may have been because of the high tolerance of primary cells to LPS and species specificity. However, incubation of cells with 30  $\mu\text{g/ml}$  LPS for 12 h significantly reduced cell vitality and induced modality change, and therefore we selected this concentration and time as the experimental conditions in this study.

Clinical treatment of inflammation caused by bacterial infection and its associated symptoms involves the use of antibiotic drugs (Ugurlu *et al.*, 2010; Pandrea *et al.*, 2016). However, the use of these drugs is associated with severe side effects (Kumar *et al.*, 2016; Yoon *et al.*, 2016), indicating the need for natural methods of inflammation control. Punicalagin, a large natural polyphenolic compound found in pomegranates, is a traditional medicine reported to



**Fig. 6 Effect of punicalagin on LPS-induced MAPK activation**

Cells were pretreated with punicalagin (5, 10, and 20  $\mu$ g/ml) for 2 h, exposed to 30  $\mu$ g/ml LPS for 15, 45, and 90 min, and analyzed by Western blotting. Phosphorylation levels of p38 (a), JNK (b), and ERK (c) were analyzed using phospho-specific anti-p38, phospho-specific anti-JNK, and phospho-specific anti-ERK antibodies. Data represent the mean  $\pm$  SEM of three independent experiments. ###  $P < 0.001$  vs. the control (Con) group. \*  $P < 0.05$ , \*\*\*  $P < 0.001$  vs. the model (Mod) group. Con: control; Mod: treated with LPS (30  $\mu$ g/ml) only; Low: punicalagin (5  $\mu$ g/ml)+LPS (30  $\mu$ g/ml); Mid: punicalagin (10  $\mu$ g/ml)+LPS (30  $\mu$ g/ml); High: punicalagin (20  $\mu$ g/ml)+LPS (30  $\mu$ g/ml)

exhibit anti-inflammatory activity (Jean-Gilles *et al.*, 2013; Olajide *et al.*, 2014; Peng *et al.*, 2015; Yaidikar and Thakur, 2015). Jean-Gilles *et al.* (2013) showed that punicalagin may be toxic to cattle and rats *in vivo*. In this study, punicalagin did not have toxic effects on bEECs at concentrations of 0–30  $\mu$ g/ml. These results are consistent with those of Kulkarni *et al.* (2007) showing that punicalagin is toxic only at higher concentrations in cells of Vero (a normal African green monkey kidney cell line), Hep-2 (a human larynx epithelial cancer cell line), and A-549 (a human small cell lung carcinoma cell line). This indicates that the inhibition of LPS-induced inflammatory injury in bEECs was not a result of toxicity.

*E. coli* induces a rapid inflammation response in endometrial epithelial cells, which release a large number of pro-inflammatory cytokines, including IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$  (Chapwanya *et al.*, 2013; Huang *et al.*, 2016). These cytokines attract immune effector cells to fight infection; however, excessive expression of these cytokines injures the cells. In this study, the potential effect of punicalagin on the expression of pro-inflammatory cytokines

(IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$ ) was analyzed by RT-PCR. As expected, the expression of IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$  was upregulated at all time points after LPS stimulation. Punicalagin inhibited the expression of IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$  in a dose-dependent manner, which protected bEECs from LPS.

NF- $\kappa$ B plays a central role in regulating immune and inflammatory processes, and thus is a target in developing novel treatments for inflammatory diseases (Checker *et al.*, 2012; Ling *et al.*, 2012). Before activation, NF- $\kappa$ B is bound in the cytoplasm by I $\kappa$ B $\alpha$ , an inhibitory protein that keeps NF- $\kappa$ B in an inactive state. When activated by various stimulants such as LPS, I $\kappa$ B $\alpha$  is phosphorylated by I $\kappa$ B kinase (IKK) and is separated from NF- $\kappa$ B. Free NF- $\kappa$ B then translocates from the cytoplasm into the nucleus, where it binds specifically to certain DNA sequences, promoting the expression of specific target genes (Corbetta *et al.*, 2005; Gu *et al.*, 2012). Previous studies reported that treatments of BV2 and THP1 cells with LPS greatly upregulated the phosphorylation level of NF- $\kappa$ B p65, indicating activation of the NF- $\kappa$ B signal pathway (Lim *et al.*, 2012; Wang *et al.*,

2013). To confirm the specific effect of punicalagin on NF- $\kappa$ B activity in bEECs, Western blotting was performed to detect levels of I $\kappa$ B $\alpha$  and phosphorylated p65. Punicalagin significantly inhibited LPS-induced NF- $\kappa$ B signaling in bEECs by suppressing the degradation of I $\kappa$ B $\alpha$  and the phosphorylation of p65 within 15, 45, and 90 min of LPS stimulation. We therefore propose that punicalagin prevented inflammation in the LPS-activated bEECs through the NF- $\kappa$ B pathway.

The TLR-4-mediated NF- $\kappa$ B pathway is well established as a signaling pathway responsible for inflammatory responses. In addition to NF- $\kappa$ B activation, TLR-4 can initiate MAPK signaling (Risco *et al.*, 2012; Hines *et al.*, 2013). Phosphorylation of MAPK family members including ERK, p38, and JNK activates a series of transcription factors, such as activator protein 1 (AP-1), cAMP-response element binding protein (CREB), and signal transducers and activators of transcription (STAT), and subsequently promotes transcription of cytokines (Morimoto *et al.*, 2009; Ruimi *et al.*, 2010). Punicalagin has been shown to inhibit the expression of pro-inflammatory genes by regulating the phosphorylation of proteins of the MAPK pathway (Olajide *et al.*, 2014; Xu *et al.*, 2014). However, whether the MAPK signaling pathway undergoes any changes in bEECs had not been determined. In the current study, LPS enhanced the phosphorylation levels of p38, JNK, and ERK in bEECs, but these effects were markedly downregulated by punicalagin in a dose- and time-dependent manner. These results indicate that attenuation of p38, JNK, and ERK activation may be involved in the reduction of cytokine production by punicalagin.

## 5 Conclusions

This study demonstrated the protective effect of punicalagin on LPS-induced inflammatory injury in bEECs. This effect was at least partly achieved by the decreased production of pro-inflammatory cytokines mediated by reducing the degradation of I $\kappa$ B $\alpha$  and phosphorylation of p65, p38, JNK, and ERK in the NF- $\kappa$ B and MAPK pathways. However, other possible pathways and targets related to the anti-inflammatory effect of punicalagin in bEECs need to be researched in the future.

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## Compliance with ethics guidelines

An LYU, Jia-jia CHEN, Hui-chuan WANG, Xiao-hong YU, Zhi-cong ZHANG, Ping GONG, Lin-shu JIANG, and Feng-hua LIU declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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## 中文概要

**题目:** 安石榴苷对脂多糖诱导奶牛子宫内膜上皮细胞炎症损伤的保护作用

**目的:** 评估安石榴苷对脂多糖诱导奶牛子宫内膜上皮细胞炎症损伤的保护作用, 并初步探讨其作用机制。

**创新点:** 首次证明安石榴苷对脂多糖诱导奶牛子宫内膜上皮细胞炎症损伤具有保护作用, 且此作用与核转录因子  $\kappa$ B (NF- $\kappa$ B) 和丝裂原活化蛋白激酶 (MAPK) 信号通路的抑制相关。

**方法:** 用不同浓度的脂多糖 (1、10、30、50 和 100  $\mu$ g/ml) 刺激奶牛子宫内膜上皮细胞 3、6、9、12 和 18 h, 筛选出建立炎症损伤的最佳作用浓度和时间。

安石榴苷预处理细胞 2 h 后用脂多糖刺激 12 h, 用逆转录聚合酶链式反应 (RT-PCR) 检测炎症因子白细胞介素 1 $\beta$  (IL-1 $\beta$ )、白细胞介素 6 (IL-6)、白细胞介素 8 (IL-8) 及肿瘤坏死因子  $\alpha$  (TNF- $\alpha$ ) 的表达。用蛋白质免疫印迹试验 (Western blotting) 的方法检测核因子  $\kappa$ B 抑制蛋白  $\alpha$  (I $\kappa$ B $\alpha$ )、磷酸化的 p65、p38、c-Jun 氨基末端激酶 (JNK) 和细胞外调节蛋白激酶 (ERK) 的表达水平。

**结论:** MTT 结果显示, 30  $\mu$ g/ml 脂多糖刺激奶牛子宫内膜上皮细胞 12 h 能够造成细胞活力下降和形态改变 (图 2 和 3)。RT-PCR 结果显示, 安石榴苷预处理后炎症因子显著降低 (图 4)。Western blotting 结果显示, 安石榴苷预处理能显著抑制 I $\kappa$ B $\alpha$  降解以及 p65、p38、JNK 和 ERK 的磷酸化表达水平 (图 5 和 6)。综上所述, 安石榴苷对脂多糖诱导奶牛子宫内膜上皮细胞炎症损伤具有保护作用, 在治疗奶牛子宫内膜炎中具有重要价值。

**关键词:** 奶牛子宫内膜上皮细胞; 炎症因子; 炎性损伤; 安石榴苷