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LP induced/mediated PGE_2 synthesis through activation of the *ERK/NF-κB* pathway contributes to inflammatory damage triggered by *Escherichia coli*-infection in bovine endometrial tissue

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ABSTRACT

The bovine endometrium is constantly challenged with pathogenic bacteria, especially with *Escherichia coli*. In previous studies, we showed that prostaglandin E_2 (PGE_2) synthesis was increased in *E. coli*-infected bovine endometrial tissue, which promoted the development of inflammatory damage. However, the molecular mechanism underlying this accumulation of PGE_2 remained undefined. Lipoprotein (LP) is one of critical outer membrane protein in *E. coli*, which regulates inflammatory response. In this study, we determined the role of LP in PGE_2 accumulation in bovine endometrial tissue by infecting the tissue with wild endometrial pathogenic *E. coli* and *E. coli* LP deletion mutant (JE5505) strains. We demonstrate that JE5505 was less effective than pathogenic *E. coli* in inducing the production of PGE_2, *IL-6*, *TNF-α*, *HMGB-1*, and *HABP1* and that the induction of cytokines was dependent on the activation of *MAPKs*, as revealed by rapid phosphorylation of *ERK1/2/NF-κB* in the endometrial tissues, furthermore, LP also induced PGE_2 synthesis and cytokine secretion. Additionally, *ERK* and *NF-κB* inhibitors significantly inhibited PGE_2 production and cytokine secretion and reduced or attenuated tissue damage in JE5505-infected and LP induced endometrial tissues. What is more important, we reported PGE_2 introduction increased the expression of pro-inflammatory factors and DAMPs in *E. coli*-infected bovine endometrial tissue. Taken together, these results indicate that LP is involved in the accumulation of PGE_2 through the activation of the *ERK/NF-κB* pathway that induces the production of pro-inflammatory factors and damage-associated molecular patterns (DAMPs) in *E. coli*-infected bovine endometrial tissue. These results should help in better understanding and management of postpartum inflammatory diseases in dairy cows.

1. Introduction

The endometrium of postpartum dairy cows is continually subjected to physiological changes (such as, self-repair) and constant challenge with pathogenic bacteria (causing, for example, tissue damage) (LeBlanc, 2014). In the first two weeks after calving, approximately 90% bovines are observed to have bacterial contamination of uterus (Sheldon et al., 2014), which causes uterine disease including endometritis that is refractory to currently available treatments and leads to infertility and economic loss (Donofrio et al., 2008). The gram-negative bacterium, *Escherichia coli*, is the most prevalent pathogen causing endometritis in bovines, which is characterized by inflammation, tissue damage, and necrosis it is followed by anaerobic bacteria in causing pathogenicity of the uterus (Potter et al., 2010). Prostaglandin E_2 (PGE_2) plays a crucial role in endometrial inflammatory damage (Li et al., 2018); it accumulates in endometrial tissues infected with pathogens and enhances the expression of pro-inflammatory factors and damage-associated molecular patterns (DAMPs) that contribute to the inflammatory damage (Li et al., 2018). However, the mechanism underlying the role of PGE_2 accumulation in *E. coli*–infected endometrium remains unclear.

Accumulating evidence indicates that PGE_2 production/biosynthesis during inflammation is primarily regulated by cyclooxygenase (COX)-2 and microsomal PGE synthase (mPGES)-1 and is induced by a range of pro-inflammatory factors, such as interleukin-1β (*IL-1β*), tumor necrosis factor-α (*TNF-α*), and interleukin-6 (*IL-6*) (Pecchi et al., 2009;

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Stichtenoth et al., 2001). Recently, it is reported that PGE_2 was pathologically accumulated by upregulation of *COX-2* and *mPGES-1* in *E. coli*-infected bovine endometrial tissue in vitro, and promoted inflammatory factors and DAMPs (*HMGB-1* and *HABP1*) expression, which regulated the inflammatory process resulting in aggravating inflammatory damage (Li et al., 2018). However, the mechanism of PGE_2 accumulation has not been fully elucidated in *E. coli*-infected bovine endometrial tissue.

The components of the outer membrane protein of *E. coli* contain lipoproteins (LP) and lipopolysaccharides (LPS), they stimulate pathogen recognition receptors and trigger inflammatory response (Cohen et al., 2017; Zhang et al., 1997). Furthermore, LPS contributes to the pathogenic processes leading to postpartum metritis and endometritis in cattle, causing inflammation of the endometrium (Barlund et al., 2008; Cronin et al., 2012). Previous studies have revealed that LPS binds to TLR4 to activate the downstream intracellular signaling mitogen-activated protein kinases (*MAPK*) and transcription factor NF-kappa-B (*NF-κB*) pathways, which lead to gene transcription and production of inflammatory mediators, including prostaglandins, cytokines, and chemokines in bovine endometrial epithelial and stromal cells (Cronin et al., 2012; Davies et al., 2008; Swangchan-Uthai et al., 2012; Turner et al., 2012). Importantly, the expression levels of *COX-2* and PGE_2 production are controlled by *MAPK-ERK*, *NF-κB*, and *AP-1* signaling (Hu et al., 2012; Hayashi et al., 2014; Mc et al., 2012; Xia et al., 2015). However, the relationship between PGE2 and LP is not yet clear in *E. coli*-infected bovine endometrium. It is reported that LP induce the production of inflammatory factors *IL-6* and *TNF-α* (Zhang et al., 1997). In addition, *Mycobacterium tuberculosis* surface LP, MPT83 (Rv2873), facilitates *M. tuberculosis* infection, and also induces the production of cytokines in mouse macrophages (Lu et al., 2013; Chen et al., 2012). Furthermore, MPT83 induced *COX-2* expression via *TLR2/NF-κB* in macrophages and induced apoptosis in both human and mouse macrophages (Wang et al., 2017). The up-regulation of *COX-2* expression promotes PGE_2 production, which increases the inflammatory response stimulating the simultaneous secretion of pro-inflammatory cytokines and various chemokines, leading to endometrial tissue damage (Sheldon et al., 2014). Therefore, a vicious circle in which *E. coli*-infected bovine endometrial tissue leads to the secretion of PGE_2 followed by aggravation of tissue damage by PGE_2, which causes further endometrial tissue damage and inflammation, has been suggested to exist.

Based on these observations, dose *E. coli* outer membrane LP like LPS, activates the *MAPK/ERK* and *NF-κB* signaling, and thereby, induces the production of PGE_2 enhanced upon uterine *E. coli*-infected bovine endometrial tissue infection. A better understanding of these PGE_2-related mechanisms would provide a theoretical framework for the rational application of non-steroidal anti-inflammatory drugs or prostaglandins in the treatment of bovine infertility as a result of endometritis.

2. Materials and methods

2.1. Chemicals, reagents, and antibodies

The following reagents and supplies were purchased from the indicated manufacturers: *COX-2* inhibitor NS-398 and *mPGES-1* inhibitor MK886 (Cayman chemical, Ann Arbor, MI, USA), *ERK* inhibitor and *NF-κB* inhibitor (SIGMA, Munich, Germany), Pam3CSK-synthetic diacylated lipopeptide (InvivoGen, San Diego, CA, USA), Fetal bovine serum (ExCell Biology, Inc., China), DMEM/F-12 (Gibco, Grand island, USA), penicillin and streptomycin (Gibco, Carlsbad, USA), Amphotericin B (GENERAY, Shanghai, China), 6-well culture plates (Corning, New York, USA), T-PER Tissue Protein Extraction Regent (Thermo Fisher Scientific, Rockford, USA), Halt Protease Inhibitor (Thermo Fisher Scientific, Rockford, USA), SDS-PAGE Loading Buffer (TAKARA, Otsu, Japan), Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, USA), prestained protein ladder (Thermo Fisher Scientific, Rockford, USA), centrifugal filter units (Millipore, USA), SDS-PAGE gel electrophoresis kit (Solarbio, Beijing, China), Trizma base (SIGMA, Munich, USA), SDS (GENERAY, Beijing, China), 10 × Tris/Glycine Buffer (Bio-Rad, California, USA), transfer membranes (Millipore, Massachusetts, USA); Starting Block T20 (TBS) Blocking Buffer (Thermo Fisher Scientific, Rockford, USA), Antibody diluent (Beyotime, Shanghai, China), AxyPrep Multisource Total mRNA Miniprep Kit (Axygen Scientific, Suzhou, China), Primer Script RT Master Mix (TAKARA, Kusatsu, Japan), SYBR Green Master (Rox) (Roche, Mannheim, Germany), Luria Bertani Broth (Oxoid, Hampshire, England), and optimal cutting temperature (O.C.T.) compound (Sakura, Torrance, USA). All antibodies used in this study are listed in Table 1.

Table 1
Antibodies used in this study.

Antibody	Lot number	Company
COX-2	ab23672	Abcam
mPGES-1	160140	Cayman
ERK	9910	Cell Signaling Technology
NF-κB	9926	Cell Signaling Technology
HMGB-1	NB100-2322	Novus Bio
HABP1	abx104799	Abbexa
Goat anti-rabbit IgG HRP-linked	7074	Cell Signaling Technology
Goat anti-mouse IgG HRP-linked	7076	Cell Signaling Technology
Goat anti-rabbit IgG H&L antibody	ab150075	Abcam

2.2. Collection and in vitro cultivation of endometrial tissue

All animal experiments were conducted in accordance with the experimental practices and standards approved by the Animal Welfare and Research Ethics Committee of Inner Mongolia Agricultural University. All efforts were made to minimize animal suffering. Fresh bilateral pro-estrus uterine horns (close to the ovary) from 20 healthy Holstein dairy cows (age: 24 months; weight: ˜600 kg) were obtained from a local abattoir. All tissues were kept on ice for transport to the laboratory and until further processing. The tissues were first washed three times with sterile phosphate-buffered saline (PBS), supplemented with 100 IU/mL penicillin, 100 IU/mL streptomycin, and 2.5 μg/mL amphotericin B. They were then incubated at 4 °C for 1 h to eliminate possible contamination and filter tissue fragments from target tissues in the test operation as the endometrial tissues were minced into pieces. Each uterine horn was excised under aseptic conditions and opened longitudinally. The endometrial tissue containing epithelial and stromal cells was removed from the endometrial region using curved scissors and ophthalmic tweezers. The endometrial tissues were minced into pieces (˜2 mm in diameter and ˜1 mm in thickness) (Li et al., 2018). These tissues were randomly placed in separate wells of 6-well plates. Each well contained 5 mL culture medium (DEME/F-12), supplemented with 20% fetal bovine serum, 100 IU/mL penicillin, 100 IU/mL streptomycin, and 2.5 μg/mL amphotericin B). The endometrial explants were incubated in a humidified environment (5% CO_2/95% O_2) at 37 °C. The culture medium was replaced every 24 h. To eliminate environmental stress, the treatment began 48 h after the excision, which showed no degradation without any treatment stained with H&E.

2.3. Preparation of bacterial suspension

Pathogenic endometrial E. coli infecting the endometrium was isolated from the uteri of bovines with clinical endometritis (Identification certificate number: SYS110017), exhibiting characteristics of tissue damage, necrosis, and accumulation of pus in the genital tract. These *E. coli* cells and those of an *E. coli* LP deletion mutant (JE5505) were cultured overnight in Luria-Bertani Broth containin, at 37 °C with

shaking at 200 rpm, until the mid-logarithmic growth phase. The culture was then centrifuged at 6000 × *g* for 10 min at 4 °C. The resulting pellet was washed in sterile PBS and resuspended in the tissue culture medium.

2.4. Experimental treatments

The medium used for culturing the bovine endometrial fragments was replaced with 5 mL DEME/F-12 containing 20% fetal bovine serum. Endometrial fragments were then treated in the following groups: a control group (without the endometrial pathogenic *E. coli* and *E. coli* mutant strain), an endometrial pathogenic *E. coli* group, a JE5505 group, an *E. coli* + *ERK* inhibitor group, an *E. coli* + *NF-κB* inhibitor group, a JE5505 + *ERK* inhibitor, and a JE5505 + *NF-κB* inhibitor group. Live endometrial pathogenic *E. coli* and JE5505 cells (1×10^6 colony forming units (CFU)/mL) were added to all the non-control wells. The concentration of *ERK* and *NF-κB* inhibitor are 10^{-5} M and 10^{-6} M, which were respectively determined by pre-experiment. All the plates were then incubated for 10 h.

The collected explants were washed with PBS, snap-frozen in liquid nitrogen, and stored at −80 °C until mRNA and protein extraction was carried out at the indicated times. One part of explants were soaked in O.C.T. compound and stored at −80 °C prior to the immunofluorescence analysis, the other part were fixed in 4% paraformaldehyde and paraffin sections were made for H&E staining.

2.5. Real-Time RT-PCR analysis

Total mRNA extraction, reverse transcription, and real-time RT-PCR were conducted according to the manufacturer's instructions. cDNA was amplified as follows: denatured at 50 °C for 2 min, 95 °C for 10 min, subjected to 40 cycles of 95 °C for 15 s, 60 °C for 30 s (ABI Quantstudio™7). The primers were synthesized by Invitrogen, China. *β-actin* was used for housekeeping genes in the present study. Because the expression and activity of β-actin were stabilized in cells, which not be affected by external factors. Differences in gene expression were calculated using the $2^{-\triangle\triangle Ct}$ method. All primers used for RT-PCR are listed in (Table 2)

2.6. ELISA

The concentrations of PGE_2, IL6, and *TNF-α* were measured in tissue culture supernatants using ELISA (Prostaglandin E2 ELISA Kit-Monoclonal item: 514010 from Cayman; Bovine *IL-6* ELISA Reagent Kit DY8190 from R&D Systems, Bovine TNF-alpha Duo Set DY2279 from R &D Systems Europe Ltd.), following the manufacturers' instructions.

2.7. Western blot analysis

Total protein extraction was performed on ice (30 min) using T-PER, and the determination of concentration and protein denaturation were conducted according to the manufacturer's instructions. The denatured proteins were stored at −80 °C. Equal amounts (20 μg) of proteins were electrophoresed through 12% SDS-polyacrylamide gel at 100 V. The proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane using semi-dry blotting at 40 V for 40 min. The membrane was blocked for 2 h at room temperature (RT) with 3% BovineSerum Albumin (BSA) in TBST (Tris-buffered saline supplemented with 0.1% Tween 20), followed by incubation in primary antibody at 4 °C for 10 h. The primary antibodies used were: *COX-2* (1:1000), *mPGES-1* (1:200), *ERK* (1:1000), p*ERK* (1:2000), *NF-κB* (1:1000), p*NF-κB* (1:1000), *HMGB-1* (1:1000), and *HABP1* (1:200). After incubation, the membranes were washed with TBST, and incubated in 1:7500 dilutions of the secondary antibody at RT for 40 min. After incubation, the membranes were washed three times with TBST at RT (45 min for each wash), and the protein bands were visualized by enhanced chemiluminescence. The band densities were quantified using the ImageJ software. The band density of the target proteins was normalized based on the density of β-actin band obtained for the same samples.

2.8. Double-label immunofluorescence assays

Frozen sections (6 μm) of treated endometrial explants were thawed at RT for 15 min and fixed in cold acetone for 10 min. The thawed sections were washed in cold PBS (with 0.25% Tween), and blocked for 1 h in 3% BSA at RT. Two primary antibodies [*HMGB-1* (1:200) and *HABP1* (1:20)] were added, and the sections were incubated overnight at 4 °C in darkness. After incubation, the slides were washed three times for 15 min each using PBS (with 0.25% Tween), and then incubated in a 1:1000 dilution of the secondary Donkey Anti-Rabbit IgG H&L antibody (Alexa Fluor 647) for 1 h at RT.

2.9. H&E staining analysis

Endometrial explants were immersed in 4% paraformaldehyde for 4 h, then transferred to 70% ethanol. Individual lobes of endometrial explants biopsy material were placed in processing cassettes, dehydrated though a serial alcohol gradient and embedded in paraffin wax block. Before immunostaining, lung tissue sections 5 um endometrial explants sections were dewaxed in xylene, rehydrated by decreasing the concentration of ethanol, and washed in PBS, and then stained with hematoxylin and eosin. After staining, the sections were dehydrated by increasing the concentration of ethanol and xylene.

Table 2
Primer sequences for qPCR.

Gene name	Generyay ID	Seqμence (5'-3')	Tm (℃)	Concentration (nM)
β-actin	J170508C59	F: GTGACAGCAGTCGGTTGGAT R: CTTAGAGAGAAGCGGGGTGG	54	400
COX-2	J161027A01	F: GGTGCCTGGTCTGATGATGT R: GATTAGCCTGCTTGTCTGGAAC	56	400
mPGES-1	J161028C79	F: ATGGTACACACCGTGGCATA R: CACAATCTCAAAGGGCCATC	55	400
IL-6	J170305C20	F: ATGCTTCCAATCTGGGTTC R: TGAGGATAATCTTTGCGTTC	52	400
TNF-α	J170305C13	F: ACGGGCTTTACCTCATCTACTC R: GCTCTTGATGGCAGACAGG	56	400
HMGB-1	J161028C72	F: AATCAAGGCGAACATCCTG R: ATCCGCAGCAGTGTTATTCC	54	400
HABP1	J181226A90	F: TTGTCTCCATCTGGGTTTCTG R: CTACGTTGACGACGGCTACTC	60	400

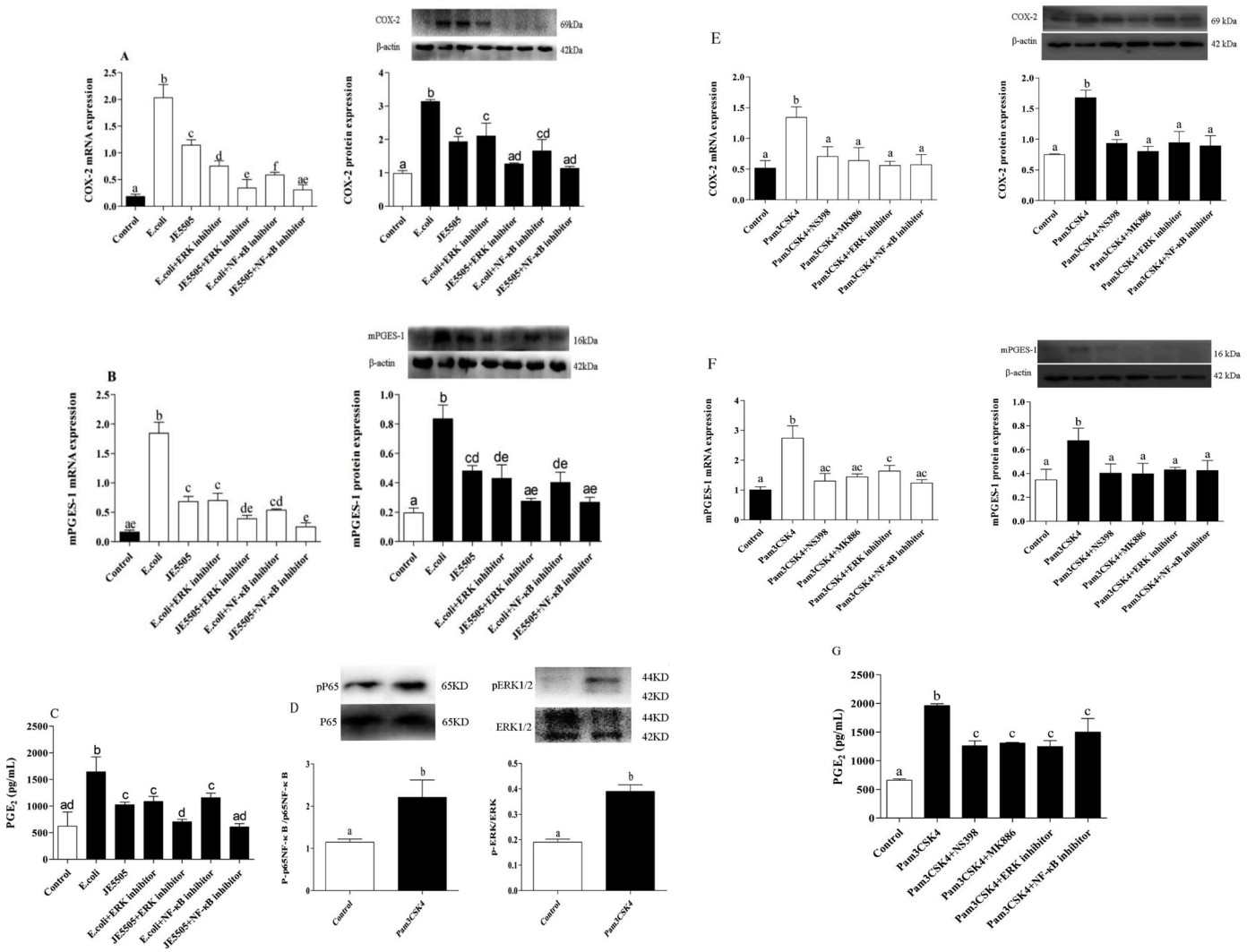

Fig. 1. LP induced PGE_2 accumulation in *E. coli* infected bovine endometrial explants. The mRNA and protein expression of (A) *COX-2* and (B) *mPGES-1* in bovine endometrial explants treated with *ERK* inhibitor or *NF-κB* inhibitor in the presence of *E. coli* or JE5505 in vitro, as detected by real-time RT-PCR and western blot analyses. (C) The concentration of PGE_2 in culture supernatants of bovine endometrial explants was detected by ELISA. (D) Phosphorylation of p65 and *ERK*1/2 was detected by western blot analysis. The mRNA and protein expression of (E) *COX-2* and (F) *mPGES-1* in bovine endometrial explants treated with *COX-2*, *mPGES-1*, *ERK* and *NF-κB* inhibitors in the presence of Pam3CSK in vitro, as detected by real-time RT-PCR and western blot analyses. (G) The concentration of PGE_2 in culture supernatants of bovine endometrial explants was detected by ELISA.

2.10. Statistical analysis

All numerical data are presented as means ± SEMs. The statistical significance was determined with one-way analysis of variance (ANOVA), followed by a post-hoc analysis (Dunnett's test) to control for the number of comparisons (n = 4). A value of $P \leq 0.05$ was considered to be statistically significant. ImageJ and GraphPad Prism 5 software were used to visualize the results.

3. Results

3.1. LP induced PGE2 accumulation in E. coli infected bovine endometrial explants

In the present study, to clearly understand the role of LP in *E. coli*-infected endometrial explants, an *E. coli* mutant strain (JE5055) lacking LP and Pam3CSK were used in experiments. The mRNA and protein expression levels of *COX-2*, *mPGES-1*, and PGE_2 production were measured in endometrial explants infected with *E. coli* and JE5505. The expression of *COX-2* (Fig. 1A), *mPGES-1* (Fig. 1B), and PGE_2 production (Fig. 1C) were enhanced in both the treatment groups compared to that in the uninfected controls ($P < 0.05$); however, the induction was higher in the *E. coli*-infected groups than in the JE5505-infected groups ($P < 0.05$), which suggests that LP may contribute to the accumulation of PGE_2 in *E. coli*-infected endometrial explants. To further determine whether the *ERK/NF-κB* pathway is involved in the regulation of PGE_2 synthesis, we treated endometrial explants in the *E. coli* and JE5505-infected groups with an *ERK* or *NF-κB* inhibitor, which markedly reduced the expression of *COX-2*, *mPGES-1*, and PGE_2 production ($P < 0.05$) compared to that in the *E. coli* and JE5505-infected groups without inhibitors; more importantly, their expression was lower in the JE5505-infected groups treated with inhibitors than in the *E. coli*-infected groups treated with inhibitors ($P < 0.05$).

These results indicated that the expression of *COX-2* and *mPGES-1*, and the secretion of PGE_2 upon JE5505 infection was lower than that caused by *E. coli* infection, indicating that the LP-*ERK/NF-κB* pathway is essential for the accumulation of PGE_2 in the bacteria-infected bovine endometrial tissues in vitro. Then, it is found that LP (Pam3CSK) activated *ERK* and *NF-κB* (Fig. 1D) and upregulated the expression of *COX-2* (Fig. 1E), *mPGES-1* (Fig. 1F), and PGE_2 production (Fig. 1G) in

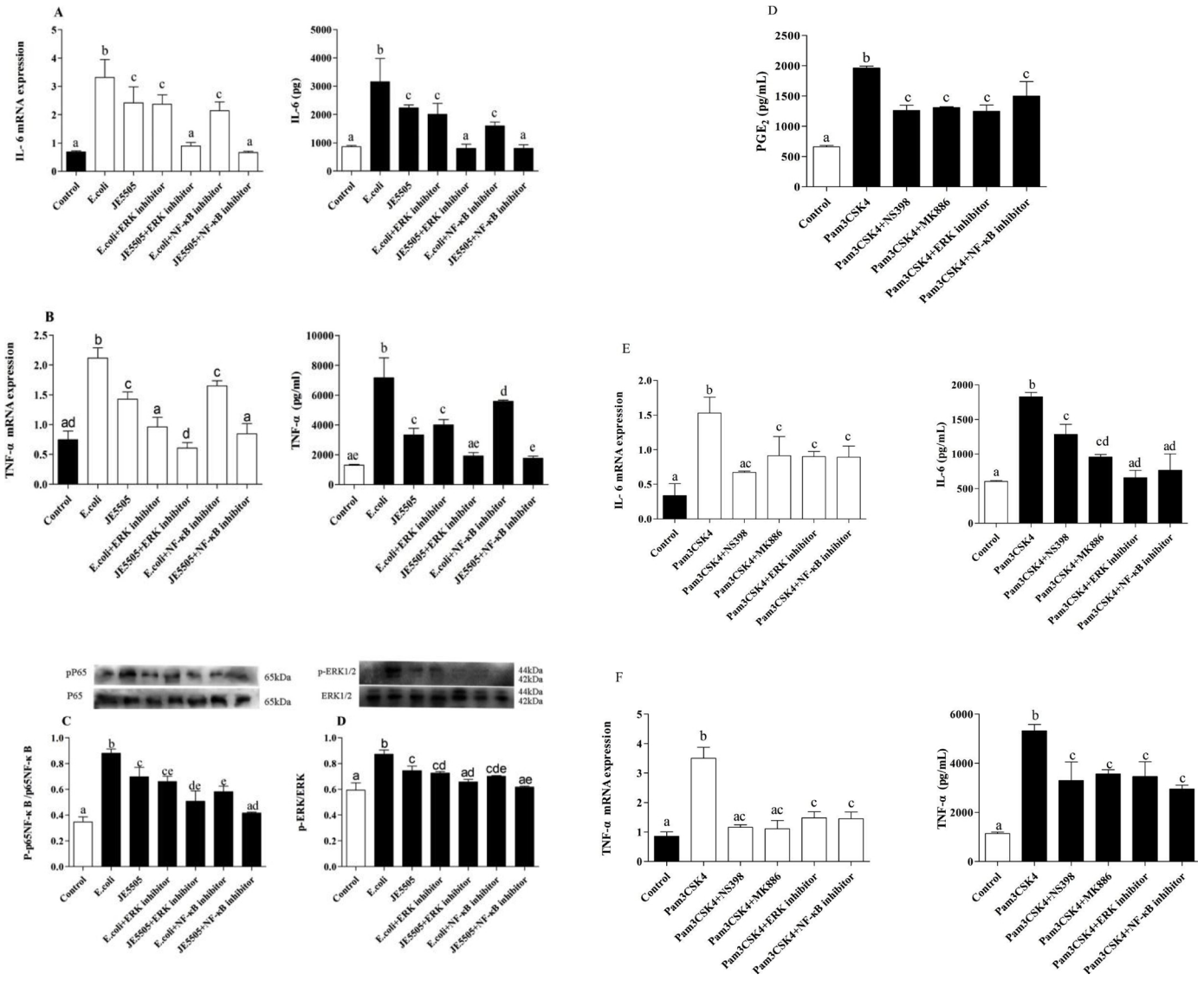

Fig. 2. LP mediated *IL-6* and *TNF-α* expression and secretion via *ERK/NF-κB* in *E. coli*-infected bovine endometrial explants. RT-PCR and ELISA results for (A) *IL-6* and (B) *TNF-α*. (C) Phosphorylation of *ERK*1/2 and (D) p65 was detected by western blot analysis. (E) *IL-6* and (F) *TNF-α* expression and secretion were detected by RT-PCR and ELISA.

endometrial explants ($P < 0.05$), while *COX-2* inhibitor (NS398) and *mPGES-1* inhibitor (MK886) reduced the induction of that induced by Pam3CSK ($P < 0.05$). In addition, *ERK* and *NF-κB* inhibitors significantly decreased PGE_2 synthesis in Pam3CSK treated with endometrial explants (Fig. 1E, F, G) ($P < 0.05$). The above results indicated that the LP-*ERK/NF-κB* pathway is essential for the accumulation of PGE_2 in *E. coli* -infected bovine endometrium.

3.2. LP mediated IL-6 and TNF-α expression and secretion via ERK/NF-κB in E. coli-infected bovine endometrial explants

To investigate whether LP is associated with that PGE_2 accumulation increased the production of pro-inflammatory cytokines in *E. coli*-infected endometrial explants. We determined the expression and secretion of *IL-6* and *TNF-α* upon bacterial infection by RT-qPCR and ELISA. As shown in Fig. 2, infection with both *E. coli* and JE5505 significantly upregulated the mRNA expression and secretion levels of *IL-6* (Fig. 2A) and *TNF-α* (Fig. 2B) compared to their levels in the uninfected control group ($P < 0.05$); however, the induction in the expression and secretion of *IL-6* and *TNF-α* were significantly less upon JE5505 infection than the induction caused by *E. coli* infection ($P < 0.05$). In addition, the induction in the expression and secretion levels of these genes by either *E. coli* or JE5505 was markedly ($P < 0.05$) prevented by the exposure of bovine endometrial explants to *ERK* and *NF-κB* inhibitors (Fig. 2A and B). On the other hand, the activation effects of *ERK/NF-κB* were significantly induced by both *E. coli* and JE5505 ($P < 0.05$, Fig. 2C and D) in bovine endometrium explants compared to that in the uninfected control group, but the activation effect of *ERK/NF-κB* was stronger in the *E. coli*-infected group than in the JE5505-infected group ($P < 0.05$). Similar results were obtained using *ERK* and *NF-κB* inhibitors (Fig. 2C and D). In addition, Pam3CSK induced *IL-6* and *TNF-α* expression in bovine endometrial explants ($P < 0.05$, Fig. 2E and F). Furthermore, *COX-2*, *mPGES-1*, *ERK*, and *NF-κB* inhibitors dramatically inhibited *IL-6* and *TNF-α* expression and secretion compared in bovine endometrial explants treated with Pam3CSK. Taken together, these results suggest that LP might trigger the expression and secretion of *ERK/NF-κB* pathway-mediated pro-inflammatory cytokines, *IL-6* and *TNF-α*, resulting in PGE_2 accumulation increasing inflammatory damage.

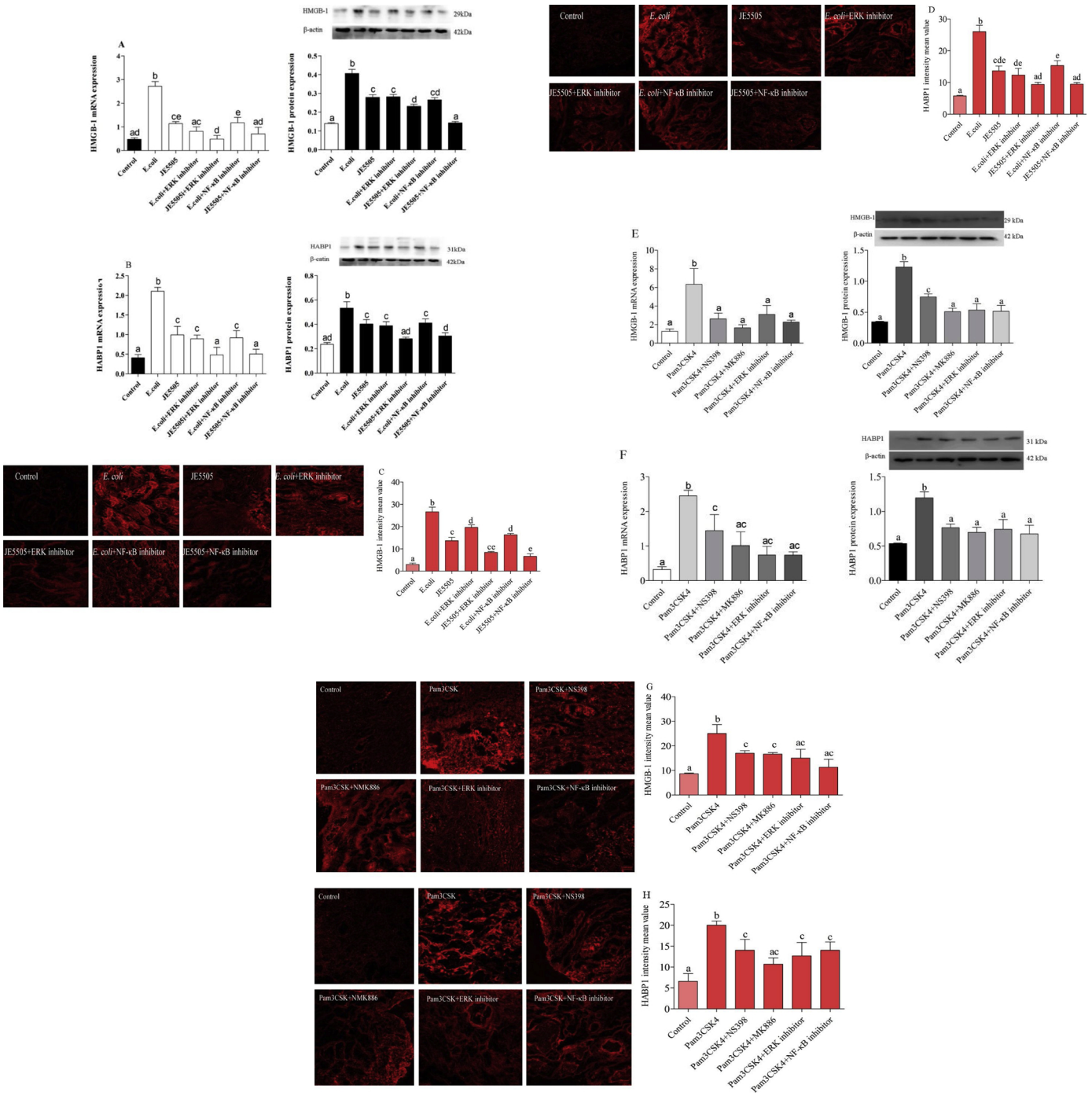

Fig. 3. Release of damage-associated molecules, *HMGB-1* and *HABP1*. Results of RT-PCR and western blot analyses for (A, E) *HMGB-1* and (B, F) *HABP1*. Immunofluorescence micrographs of bovine endometrial explant tissue sections showing (C, G) *HMGB-1* and (D, H) *HABP1* (magnification: ×100). Data are given as means ± SEMs.

3.3. Release of damage-associated molecules, HMGB-1 and HABP1

We observed that the LP-*ERK/NF-κB* pathway was essential for the accumulation of PGE_2 (Fig. 1C and G) and for the expression of *COX-2* (Fig. 1A) and *mPGES-1* (Fig. 1B). Therefore, the possible involvement of LP in inducing tissue damage was determined. The tissue damage resulting from bacterium infection by determining the expression levels of *HMGB-1* and *HABP1* were evaluated using RT-PCR, western blotting, and immunofluorescence staining. The induction of *HMGB-1* (Fig. 3A) and *HABP1* (Fig. 3B) at the mRNA and protein levels upon JE5505 infection was lower than that caused by *E. coli* infection in bovine endometrial explants ($P < 0.05$). And treatment with *ERK* and *NF-κB* inhibitors strongly inhibited the induction of *HMGB-1* and *HABP1* expression upon *E. coli* and JE5505 infection ($P < 0.05$). Additionally, the results of immunofluorescence also indicated that the fluorescent intensities obtained for *HMGB-1* (Fig. 3C) and *HABP1* (Fig. 3D) were consistent with the results of real-time RT-PCR and western blotting. Furthermore, Pam3CSK increased the expression of *HMGB-1* and *HABP1* in bovine endometrial explants than that in untreated with endometrial explants. However, *HMGB-1* (Fig. 3E) and *HABP1* (Fig. 3F) expression were dramatically inhibited by *COX-2*, *mPGES-1*, *ERK*, and *NF-κB* inhibitors in bovine endometrial explants treated with Pam3CSK

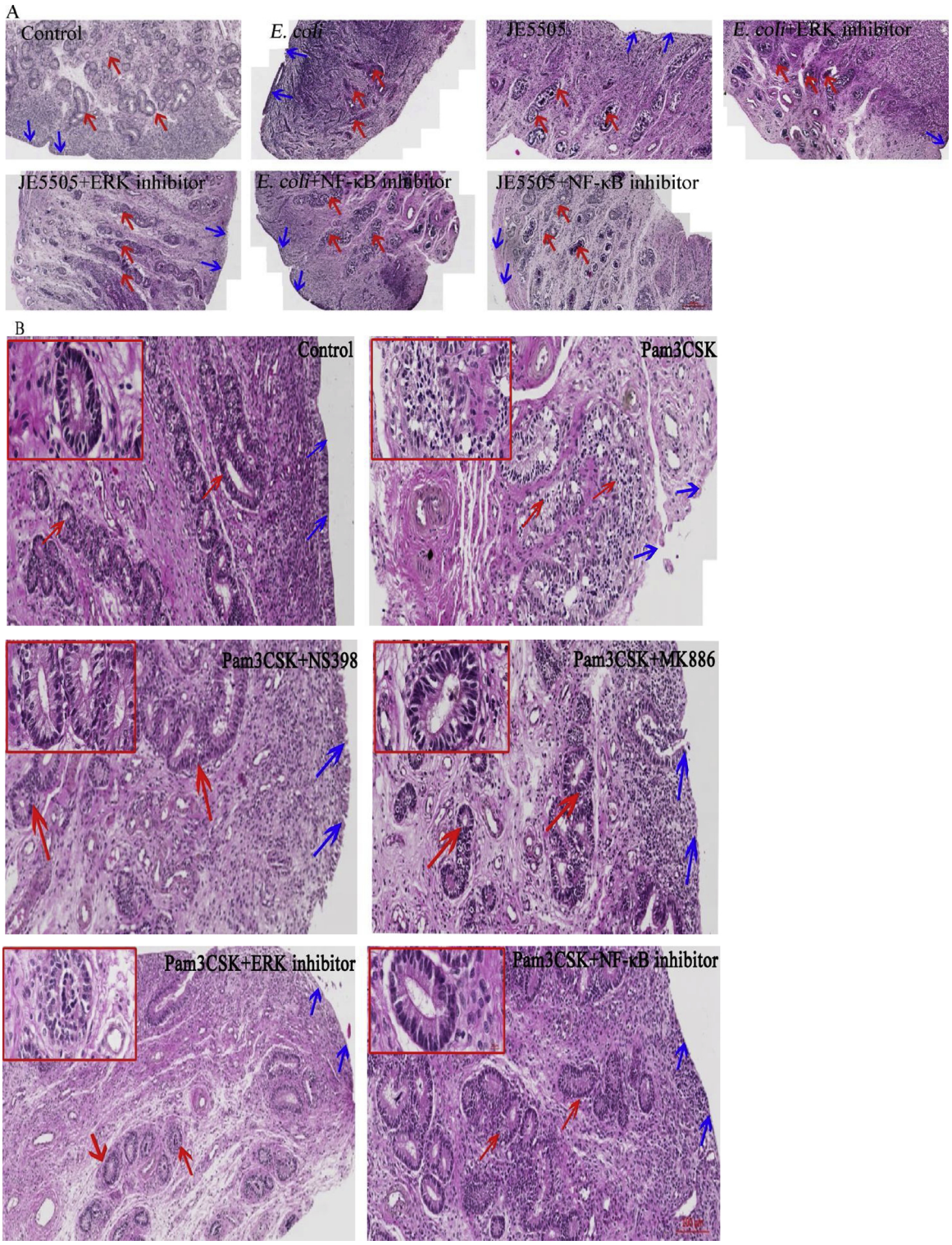

Fig. 4. LP induced endometrial tissue damage in *E. coli* infection. (A, B) Representative bovine endometrial explants stained with H&E showing tissue damage (magnification: ×100). The blue arrow refers to the endometrial epithelial cells, and the red arrow refers to the uterine gland. All the samples were infected with *E. coli* except for the control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

compared Pam3CSK group. The results of immunofluorescence (Fig. 3G and H) also show that were similar to the results of real-time RT-PCR and western blotting (Fig. 3E and F). Collectively, our data indicates that LP was involved in the mediation of tissue damage though *ERK/NF-κB* pathway in *E. coli*-infected endometrium.

3.4. LP induced endometrial tissue damage in E. coli infection

The damage done by *E. coli* infection to the endometrial tissue was assessed by H&E staining. As shown in Fig. 4A, the gross appearance of the in vitro cultured endometrial explants in the absence of any treatment was consistent with that of normal bovine endometrial tissue; the tissue architecture and cytoplasm were intact, the nucleus was also

intact with a smooth, regular surface. Moreover, longitudinal sectioning showed that the luminal epithelium and endometrial glands were undamaged and clearly visible throughout the stromal compartment of endometrium in the control group. In bovine endometrial explants infected with *E. coli*, the endometrial glands were extensively detached, and several endometrial and glandular epithelial cells had disappeared. However, the extent of damage to endometrial explants infected with JE5505 was less severe than in those infected with *E. coli*; some part of endometrial glands were clearly visible, and some epithelial and glandular epithelial cells could still be seen. Apparently, in the *E. coli* and JE5505-infected bovine endometrial explants treated with *ERK* and *NF-κB* inhibitors, the damage to the epithelial luminal layer and endometrial glands was reduced compared to that in the *E. coli* and JE5505-infected group which were not treated with the inhibitors from observing the endometrial glands and the endometrial epithelial cells. Moreover, the damage in the JE5505 infection group that was exposed to the inhibitors was less than in the *E. coli* infection group treated with the same inhibitors, the endometrial glands and majority of the endometrial epithelial cells being visible in the former group. Furthermore, Pam3CSK induced bovine endometrial explants damage including endometrial epithelial cell shedding and glands detached, however, which was alleviated by *COX-2*, *mPGES-1*, *ERK*, and *NF-κB* inhibitors, characterized by no destruction in glands although the epithelial cells remain shedding (Fig. 4B). Thus, the morphological evidence was consistent with the functional experiments.

4. Discussion

Bovine endometrium is always easily infected with bacteria after parturition, which results in endometrial diseases (Sheldon et al., 2014). For example, bovine endometritis is a mucosal disease, characterized by endometrial inflammation, tissue damage, and necrosis, which occurs as a result of infection with gram-negative and gram-positive bacteria, such as *E. coli* and *Staphylococcus* (Goldstone et al., 2014; Nishimura et al., 2013). PGE_2 acts as a double-edged sword, playing positive and negative roles in reproduction as well as in endometrial inflammation (Chapwanya et al., 2013; Huang et al., 2016; Li et al., 2018; Lyu et al., 2017;Zhang et al., 2017). PGE_2 is involved in the repair of bovine endometrium by regulating tissue growth factors and efficiently initiating the repair process (Chapwanya et al., 2013; Huang et al., 2016; Lyu et al., 2017; Zhang et al., 2017). In contrast, previous work in our laboratory has demonstrated that PGE_2 accumulates in *E. coli*-infected bovine endometrial tissue and increases inflammatory damage (Li et al., 2018). Several questions remain to be addressed in this regard; these include the identification of *E. coli* factors responsible for PGE_2 accumulation, clarification of the mechanisms through which the PGE_2 production contributes to *E. coli* infection in endometrial tissue, and determination of the exact effect of PGE_2 on the outcome of tissue damage. As expected, we found that LP was involved in PGE_2 accumulation in *E. coli*-infected bovine endometrial tissue. We also provide evidence that LP contributes to PGE_2 accumulation by activation of the *ERK/NF-κB* pathway, increasing the inflammatory damage and promoting the development of endometrial inflammation.

PGE_2 is a well-recognized pro-inflammatory mediator, generated by COX and PGES; *COX-2* and *mPGES-1* are especially vital for the pathological biosynthesis of PGE_2 in the bovine endometrium (Li et al., 2018; Murakami et al., 2000). Consistent with our results, the expression levels of *COX-2* and *mPGES-1* were reported to be increased in endometrial tissue during endometritis (Li et al., 2018), and LPS increased the levels of human monocytes and differentiated macrophages, promoting *mPGES-1* expression and PGE_2 production (Sheldon et al., 2014). In the present study, the expression levels of *COX-2* and *mPGES-1* mRNA and protein were observed to be induced in the *E. coli* and JE5505-infected endometrial tissue. Moreover, the supernatants of the endometrial explant cultures were enriched in PGE_2 when these explants were challenged with live *E. coli* and JE5505, an observation consistent with those of previous studies wherein PGE_2 production in the endometrium of cattle infected with bacterial was increased (Nishimura et al., 2013). However, it is found that the expression of *COX-2* and *mPGES-1*, and the production of PGE_2 in JE5505-infected endometrial tissues were significantly lower than in *E. coli*-infected endometrial tissues, suggesting that mutant strain lacking LP was significantly less efficient at inducing PGE_2 production. Additionally, Pam3CSK induced *COX-2* and *mPGES-1* expression and PGE_2 production in endometrial tissues. This suggests that LP might mediate PGE_2 production in bovine endometrial tissues, thereby, enhancing the endometrial inflammation. Alternatively, other outer membrane proteins of *E. coli* might stimulate the production of PGE_2 and increase the damage to endometrium.

LP, the outer membrane protein of *E. coli*, and LPS are intricately associated with the stimulation of pathogen recognition receptors and the triggering of inflammatory response (Cohen et al., 2017; Zhang et al., 1997). LPS contributes to the pathogenic processes leading to postpartum inflammation of the endometrium and tissue damage in cattle (Barlund et al., 2008; Cronin et al., 2012; Donofrio et al., 2008). In a previous study, we demonstrated that LPS induced the production of PGE_2 in endometrium in vitro. Importantly, inhibitors of the inducible enzymes in PGE_2 biosynthesis via *COX-2* and *mPGES-1*, reduced the tissue damage induced by LPS in bovine endometrium (Yang Deng, unpublished results). In addition, a number of studies have shown that LPS binds to TLR4 to activate the downstream members of the intracellular *MAPK* and *NF-κB* signaling pathways, which leads to gene transcription and production of inflammatory mediators, including prostaglandins (PG)s, cytokines, and chemokines, in bovine endometrial epithelial and stromal cells (Cronin et al., 2012; Davies et al., 2008; Swangchan-Uthai et al., 2012; Turner et al., 2012). In the present study, infection with live *E. coli* mutants also increased PGE_2 production and expression of *IL-6*, *TNF-α*, HMGB1, and *HABP1*, suggesting that in addition to LP, LPS or other factors mediate PGE_2 production in *E. coli*-infected endometrial tissues. Mc Elroy et al. (Mc et al., 2012) reported that *COX-2* expression and PGE_2 production are controlled by *MAPK/ERK*, *NF-κB*, and AP-1 signaling (Braun, 1975; Chen et al., 2012; Kim et al., 2017; Lu et al., 2013; Wang et al., 2017). In the present study, *ERK* inhibitor and *NF-κB* inhibitor greatly diminished the expression of *COX-2*, *mPGES-1*, *IL-6*, *TNF-α*, HMGB1, and *HABP1*, as well as the production of PGE_2 in JE5505- infected, *E. coli*-infected, and Pam3CSK induced endometrial tissues, but the inhibitory effects induced by bacterium were higher in the *E. coli*-infected groups than in the JE5505-infected groups. Possibly, both LPS and LP might induce the production of PGE_2 through *ERK/NF-κB* signaling pathway and aggravate the tissue damage.

It is very important to note that LP stimulates the pathogen recognition receptors and triggers inflammatory response in *E. coli*, *M. tuberculosis*, and *Streptococcus gordonii* infections (Kim et al., 2017; Wang et al., 2017; Zhang et al., 1997). However, little is known about the response of PGE_2 production to LP and about the signaling pathway that might be operative in *E. coli*-infected endometrial tissues. As mentioned by Hooda (Hooda and Moraes, 2018), there have been no recent studies for determining the role of LP in gram-negative bacteria with regard to PGE_2 production; we hypothesize that LP, like LPS, could also be involved in the production of PGE_2, which might contribute to the inflammatory damage triggered in *E. coli*-infected bovine endometrial tissue. As expected, because of the deficiency of LP, JE5505 infection strikingly triggered the increase of PGE_2, *IL-6*, and *TNF-α*, which were lower than in the case of *E. coli* infection. To specifically observe the damage to the endometrial tissue we detected the expression of DAMPs, HMGB1 and *HABP1*. In addition, we found that the release of HMGB1 and *HABP1* was less induced by JE5505 infection than it was in the case of *E. coli* infection. Furthermore, Pam3CSK increased PGE_2 synthesis, proinflammatory factors expression and secretion, and DAMPs expression. However, the *ERK* and *NF-κB* inhibitors alleviated the inflammatory reaction by decreasing the production of

PGE_2, and decreasing the *IL-6*, *TNF-α*, HMGB1, and *HABP1* expression in the bacteria-infected and Pam3CSK induced bovine endometrial tissues, thereby, mediating the tissue damage. These results were consistent with the observation that *Mycobacterium tuberculosis* lipoprotein, MPT83 induced the expression of *TNF-α*, *IL-6*, and *COX-2*, and promoted the apoptosis of infected macrophages by activating the phosphorylation of *ERK*1/2 and *NF-κB*.

5. Conclusions

We demonstrate that LP is an important factor underlying the accumulation of PGE_2 through the activation of *ERK/NF-κB* signaling, which contributes to inflammatory damage in *E. coli*-infected bovine endometrial tissues. Understanding LP increasing PGE_2 accumulation in *E. coli*-infected bovine endometrial tissues would help in devising strategies that can be deployed to enhance bovine health.

Author contributions

Ruifeng Gao, Shuangyi Zhang, Jindi Wu, Changqi Fu, Yang Deng, Kun Liu, and Yuan Shen designed the research; Wei Mao, and Bo Liu analyzed the data; Tingting Li and Jinshan Cao wrote the paper.

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