

Honokiol inhibits endoplasmic reticulum stress-associated lipopolysaccharide-induced inflammation and apoptosis in bovine endometrial epithelial cells

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Abstract. Honokiol (HKL) has been previously reported to exert anti-inflammatory effects in numerous diseases. However, the role of HKL in endometritis remains unclear. The present study aimed to explore and elucidate the role of HKL in a lipopolysaccharide (LPS)-induced *in vitro* model of endometritis. Bovine endometrial epithelial cells (bEECs) were pre-treated with HKL at doses of 1, 10 and 20 μ M, followed by 1 μ g/ml LPS. MTT assay was then used to detect cell viability. ELISA was utilized to measure the levels of the proinflammatory cytokines TNF- α , IL-1 β and IL-6 in bEECs culture supernatants. Reverse transcription-quantitative PCR was further performed to examine the mRNA expression levels of these cytokines. Cell apoptosis was observed by TUNEL staining and the levels of Bcl-2, Bax, cleaved caspase 3 and cleaved caspase 9 were assayed by western blotting. Western blotting was also performed to detect the expression levels of endoplasmic reticulum (ER) stress-related proteins activating transcription factor 6, CCAAT-enhancer-binding protein homologous protein, inositol-requiring enzyme 1 and cleaved caspase 12 in bEECs. LPS treatment reduced cell viability and HKL treatment improved the viability of bEECs after LPS treatment. The LPS-induced inflammatory response and apoptosis in bEECs were also inhibited by HKL treatment. Additionally, the increased expression of ER stress-related proteins induced by LPS was reversed by HKL treatment. Following stimulation with the ER stress inducer tunicamycin, it was revealed that HKL attenuated ER stress and inhibited LPS-induced inflammatory response and apoptosis in bEECs. In summary,

HKL inhibited ER stress associated with LPS-induced inflammation and apoptosis in bEECs, providing evidence that HKL can serve to be a novel agent for the treatment of endometritis.

Introduction

Endometritis is an inflammatory reaction of the uterine endometrial lining, which typically occurs due to infection (1). There are two types of endometritis, namely acute and chronic (2). The former is frequently triggered by miscarriage, abortion, parturition or ascending infection of the uterine cavity, whilst the latter is closely associated with infertility or problematic pregnancy (3,4). Initiation of the inflammatory response caused by Gram-negative bacteria that cause endometritis mainly occurs by the recognition of lipopolysaccharide (LPS) by Toll-like receptor 4 (TLR4) in the epithelial cells of the endometrium (5). It has been previously reported that treatment of primary bovine endometrial epithelial cells (bEECs) with LPS induces a rapid inflammatory response (6). LPS is considered to be a trigger of the inflammatory response in bEECs in an *in vitro* endometritis cell model (7). In addition, previous *in vivo* and *in vitro* studies have revealed that endoplasmic reticulum (ER) stress is involved in the inflammatory response and apoptosis of endometrial stromal cells and mouse uterine tissues (8,9).

Honokiol (HKL) is a natural phenol that can be extracted from the oriental herb *Magnolia officinalis* and has been demonstrated to protect against ER stress-related apoptosis in a rat model of testicular injury (10). The clinical use of *Magnolia officinalis* in traditional Chinese medicine for anti-inflammatory and antibacterial purposes has been documented in Chinese culture for >1,800 years. HKL is an active component of this extract that has also been shown to exhibit anti-inflammatory and anti-oxidant properties in previous studies of cardiovascular diseases, kidney injury and asthma (11-13). However, to the best of our knowledge, whether and how HKL may be of benefit in endometritis has not been elucidated to date.

In the present study, it was inferred that HKL may also confer beneficial effects on inflammation in endometritis. Lipopolysaccharide (LPS) is a classic endotoxin that has been

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used widely in inflammatory model establishment, including the establishment of an endometritis model (7,14). In particular, HKL has been demonstrated to exert protective effects against LPS-induced inflammation in the respiratory system and at the cellular level (15-17).

Therefore, the present study conducted an experimental analysis of the possible role of HKL in an *in vitro* model of LPS-induced endometritis, in the hope that the subsequent findings will provide another therapeutic approach to endometritis.

Materials and methods

Cell culture and treatment. BEND bovine endometrial epithelial cells (bEECs; ATCC[®] CRL-2398[™]) were acquired from American Type Culture Collection. The cells were grown in a 1:1 mixture of Ham's F12 and Eagle's Minimal Essential medium (Gibco; Thermo Fisher Scientific, Inc.) with Earle's balanced salts with 1.5 mM L-glutamine (MilliporeSigma) adjusted to contain 1.5 g/l sodium bicarbonate (MilliporeSigma) supplemented with 0.034 g/l D-valine (MilliporeSigma), 10% FBS (Biowest LLC) and 10% horse serum (Gibco; Thermo Fisher Scientific, Inc.) in an environment containing 5% CO₂ at 37°C. HKL (purity, ≥98%) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd., where its structural formula is shown in Fig. 1A.

Pre-treatment of the cells was performed with HKL at doses of 1, 10 and 20 μM for 1 h at 37°C, followed by stimulation with 1 μg/ml LPS for 12 h at 37°C. In addition, 1 μg/ml Tunicamycin (an ER stress inducer; purchased from MedChemExpress), was added into cells for 2 h at 37°C followed by LPS treatment. Cells without any treatment were considered to be the control group.

MTT assay. Cells in the logarithmic growth phase were collected. Subsequently, 100 μl cell suspension was added to each well of a 96-well plate to a final density of 1x10³ cells/well. Following corresponding treatment, the cells were incubated with 5% CO₂ for 12 h at 37°C. Next, 20 μl 0.5% MTT solution (Shanghai Aladdin Biochemical Technology Co., Ltd.) was added to each well, and the incubation was continued for another 4 h at 37°C. Subsequently, 150 μl DMSO solution (EMD Millipore) was added to each well, followed by gentle oscillation on a shaking rocker to fully dissolve the crystals. A microplate reader was used to measure the optical density at a wavelength of 480 nm.

ELISA. ELISA kits were used to detect the expression levels of TNF-α (cat. no. DY2279; R&D Systems, Inc.), IL-1β (cat. no. ESS0027; Thermo Fisher Scientific, Inc.) and IL-6 (cat. no. DY8190; R&D Systems, Inc.) in bEECs in different treatment groups. The supernatant was centrifuged at 14,000 x g for 10 min. Preparation of the solution was performed in accordance with the manufacturer's instructions. A total of 90 μl of the sample was added to each well and the wells were sealed and incubated in the dark for 120 min at 37°C. Subsequently, the plate was washed three times and dried with absorbent paper, followed by the addition of 100 μl 3,3',5,5'-tetramethylbenzidine solution and incubation in the

dark for 20 min at 37°C. After termination of the reaction, the absorbance was detected in each well at 450 nm.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the cells using TRIzol[®] reagent (Thermo Fisher Scientific, Inc.). RNA quality was examined by ultraviolet spectroscopy. Reverse transcription was performed to synthesize cDNA by PrimeScript RT Master Mix (Takara Bio, Inc.) and the reaction was incubated at 25°C for 5 min, 42°C for 30 min, 85°C for 5 min, and then maintained at 4°C for 5 min. Subsequently, PCR reactions were conducted with the SYBR Premix ExTaq kit (Takara Bio, Inc.) and the DNA was amplified for 5 min at 95°C; followed by 40 cycles of denaturation for 20 sec at 95°C, annealing for 20 sec at 55°C and extension for 20 sec at 72°C. After termination of PCR and natural cooldown to 60°C, the temperature was increased up to 95°C to denature the DNA products. The primer sequences for PCR were as follows: TNF-α forward, 5'-GCCTCCCTCTCATCAGTTCTA-3' and reverse, 5'-GGCAGCCTTGCCCTTG-3'; IL-1β forward, 5'-ACCTGTGTCTTTCCCGTGG-3' and reverse, 5'-TCATCTCGGAGCCTGTAGTG-3'; IL-6 forward, 5'-AGTTGTGCAATGGCAATCTGA-3' and reverse, 5'-CCCCAGCATCGAAGGTAGA-3'; and GAPDH forward, 5'-TGCTGTCCCTGTATGCCTCT-3' and reverse, 5'-TTTGATGTACGCACGATTT-3'. The 2^{-ΔΔC_q} method was applied for the relative quantification of the data using GAPDH as the normalization control (18).

TUNEL staining. The Colorimetric TUNEL Apoptosis Assay Kit (Beyotime Institute of Biotechnology) was purchased for the cell apoptosis assay. The cells were washed twice with Dulbecco's PBS and fixed with 4% paraformaldehyde at room temperature for 30 min in the dark. Then the cells were incubated with proteinase K for 15 min at 37°C and placed in 3% H₂O₂ for 15 min at room temperature, followed by staining with the TUNEL detection kit. A total of 50 μl labeling solution was added to the samples for incubation in the dark for 60 min at 37°C. The cells were then incubated with 0.1 ml termination solution at room temperature for 10 min before being washed three times with PBS (Thermo Fisher Scientific, Inc.). After washing, cells were co-labeled with DAPI working solution (1 μg/ml) for 10 min at 37°C. The staining could be observed after the cells were washed three times with PBS under a fluorescence microscope (Nikon Eclipse80i; Nikon Corporation; magnification, x200), and at least 10 fields per section for each sample were examined. TUNEL apoptosis rate (%) = number of TUNEL-positive podocytes/total number of podocytes x100%.

Western blotting. Protein samples were collected using IP lysis buffer (cat. no. G2038; Wuhan Servicebio Technology Co., Ltd.) and protein concentration was measured using a BCA kit (Beyotime Institute of Biotechnology). The samples were sufficiently denatured after boiling in a water bath at 100°C for 3-5 min. A total of 30 μg protein samples per well were separated by 10% SDS-PAGE and electrophoretically transferred onto PVDF membranes. After being blocked with 5% non-fat milk in TBS with 0.1% Tween-20 for 1 h at room temperature, the membranes were then incubated with the specific primary antibody [anti-Bcl-2 (1:1,000; cat. no. ab32124; Abcam),

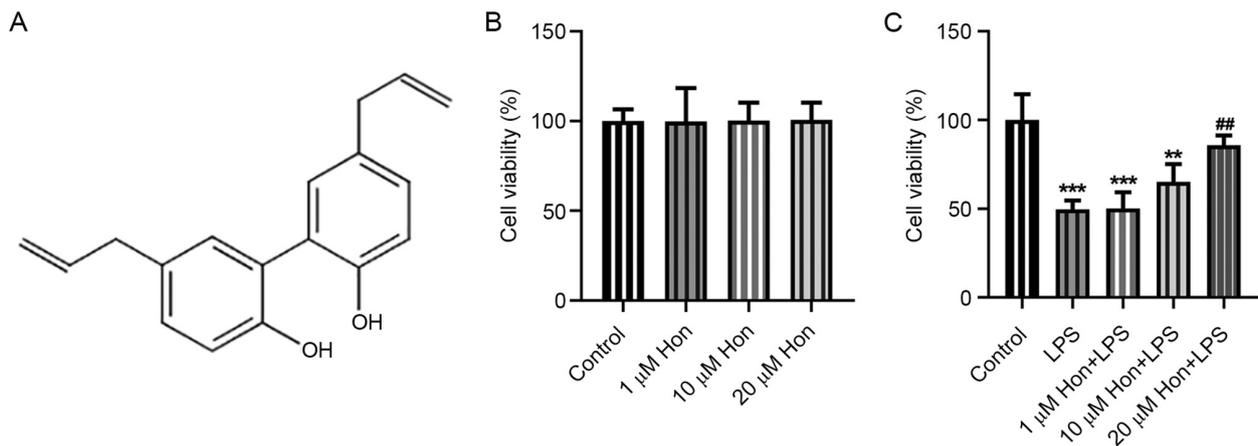


Figure 1. Honokiol treatment improves the viability of LPS-treated bEECs. (A) Chemical structural formula of HKL. (B) The viability of bEECs following treatment with different doses of HKL as detected by MTT. (C) The viability of LPS-stimulated bEECs following treatment with different doses of HKL as detected by MTT. ** $P < 0.01$ and *** $P < 0.001$ vs. Control; ## $P < 0.01$ vs. LPS. HKL or Hon, honokiol; LPS, lipopolysaccharide; bEECs, bovine endometrial epithelial cells.

anti-Bax (1:1,000; cat. no. ab32503; Abcam), anti-cleaved caspase 3 (1:500; cat. no. ab32042; Abcam), anti-cleaved caspase 9 (1:1,000; cat. no. ab2324; Abcam), anti-activating transcription factor 6 (ATF6; 1:1,000; cat. no. ab37149; Abcam), anti-CCAAT-enhancer-binding protein homologous protein (CHOP; 1:1,000; cat. no. ab11419; Abcam), anti-inositol-requiring enzyme 1 (IRE1; 1:1,000; cat. no. ab96481; Abcam), anti-caspase 12 (1:1,000; cat. no. ab8117; Abcam), anti-cleaved caspase 12 (1:1,000; cat. no. ab62484; Abcam) and anti-GAPDH (1:3,000; ab125247; Abcam) diluted with TBS-0.05% Tween-20 (TBST; Cell Signaling Technology, Inc.) on a shaking bed at 4°C overnight. The membrane was then incubated with the specific secondary antibody goat anti-mouse IgG H&L (HRP) (1:1,000; ab6789; Abcam) or goat anti-rabbit IgG H&L (HRP) (1:1,000; ab6721; Abcam) for 2 h at 37°C. TBST was used to wash the membrane before photographic development using an ECL kit (Beyotime Institute of Biotechnology) and the band density was analyzed using ImageJ software (version 1.49; National Institutes of Health). GAPDH was used as the internal reference.

Statistical analysis. GraphPad Prism 6 (GraphPad Software, Inc.) was utilized for data graphing and analysis. All data are presented as the mean \pm SD. All experiments were performed independently three times. One-way ANOVA followed by Bonferroni post hoc test was applied for the comparisons among multiple groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

HKL treatment improves the viability of LPS-stimulated bEECs. To investigate how HKL affects the viability of bEECs, cells were treated with different doses of HKL. The MTT assay results demonstrated that 0, 1, 10 and 20 μ M HKL treatment conferred no cytotoxicity to bEECs (Fig. 1B). However, after LPS induction, it was revealed that it significantly reduced bEEC viability, which was in turn improved by HKL treatment in a dose-dependent manner (Fig. 1C).

This suggest that HKL treatment may improve the viability of LPS-stimulated bEECs.

HKL treatment inhibits LPS-induced inflammation and apoptosis of bEECs. ELISA was utilized to examine the effect of HKL treatment on the inflammation and apoptosis of LPS-stimulated bEECs. Significantly increased secretion of proinflammatory cytokines (TNF- α , IL-1 β and IL-6) in bEECs was found after LPS stimulation, which were markedly suppressed by HKL treatment in a dose-dependent manner (Fig. 2A). The mRNA expression levels of these cytokines were detected by RT-qPCR, which were significantly higher in LPS-stimulated bEECs compared with those in cells in the control group. However, they were gradually decreased by treatment with increasing doses of HKL (Fig. 2B). Furthermore, TUNEL staining revealed that LPS-induced apoptosis was significantly alleviated as bEECs were treated with increasing doses of HKL (Fig. 2C and D). Western blotting also detected significantly decreased expression levels of the anti-apoptotic protein Bcl-2 and significantly increased expression levels of pro-apoptotic proteins Bax, cleaved caspase-3 and cleaved caspase-9 following LPS treatment (Fig. 2E and 2F). By contrast, these effects aforementioned were all marked reversed after HKL treatment, especially at the high dose (20 μ M; Fig. 2E and 2F). These results suggest that HKL treatment inhibited the LPS-induced inflammatory response and apoptosis in bEECs.

HKL inhibits LPS-induced ER stress in bEECs. The expression levels of ER stress-related proteins activating transcription factor 6, CCAAT-enhancer-binding protein homologous protein, inositol-requiring enzyme 1 and cleaved caspase-12 were also detected by western blotting, which revealed a significantly increase in their expression levels after LPS stimulation and a marked reversal by HKL co-treatment in a dose-dependent manner (Fig. 3). This suggested an inhibitory effect of HKL on LPS-induced ER stress in bEECs.

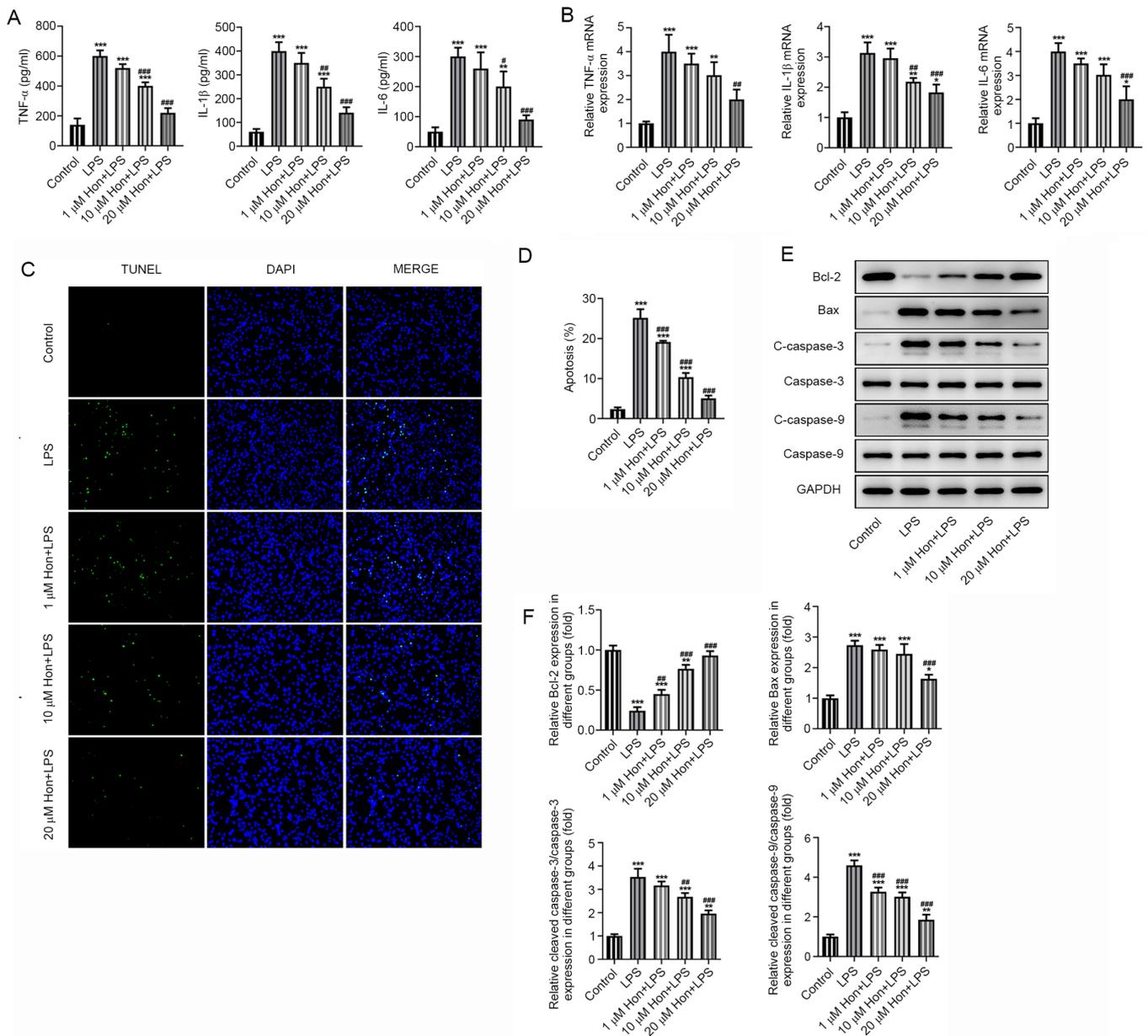


Figure 2. Honokiol treatment inhibits LPS-induced inflammation and apoptosis in bEECs. (A) The expression levels of proinflammatory cytokines TNF- α , IL-1 β and IL-6 in LPS-stimulated bEECs following treatment with different doses of HKL as detected by ELISA. (B) The mRNA expression of proinflammatory cytokines TNF- α , IL-1 β and IL-6 in LPS-stimulated bEECs following treatment with different doses of HKL as detected by reverse transcription-quantitative PCR. (C) Apoptosis level of LPS-stimulated bEECs following treatment with different doses of HKL as observed by TUNEL staining, (D) which was quantified. (E) The expression of apoptosis markers Bcl-2, Bax, cleaved caspase-3 and cleaved caspase-9 in LPS-stimulated bEECs following treatment with different doses of HKL as detected by western blotting, (F) which was quantified. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. Control. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. LPS. HKL or Hon, honokiol; LPS, lipopolysaccharide; bEECs, bovine endometrial epithelial cells.

HKL mitigates ER stress to inhibit the LPS-induced inflammatory response and apoptosis in bEECs. To identify whether ER stress is a link between HKL treatment and its effects on bEEC inflammation and apoptosis, bEECs were treated with the ER stress inducer tunicamycin (1 μ g/ml) (19), followed by treatment with 20 μ M HKL. Results from ELISA demonstrated that while the expression levels of proinflammatory TNF- α , IL-1 β and IL-6 in LPS-stimulated bEECs were significantly suppressed by HKL treatment, subsequent treatment with tunicamycin significantly reversed this effect (Fig. 4A). The same trend in the mRNA expression levels of these cytokines was also observed according to data from RT-qPCR (Fig. 4B).

Additionally, it was observed by TUNEL staining that HKL significantly reduced the apoptosis levels of LPS-stimulated bEECs, but co-treatment with tunicamycin significantly elevated these apoptosis levels again (Fig 4C and D). Consistently, western blotting revealed that tunicamycin significantly decreased the expression levels of the anti-apoptotic protein Bcl-2 whilst significantly increasing the expression levels of the pro-apoptotic proteins Bax, cleaved caspase-3 and cleaved caspase-9 in LPS and HKL-treated bEECs, compared with those in the group with LPS and HKL treatment but without tunicamycin treatment (Fig. 4E and F). These results collectively suggest that HKL inhibited the

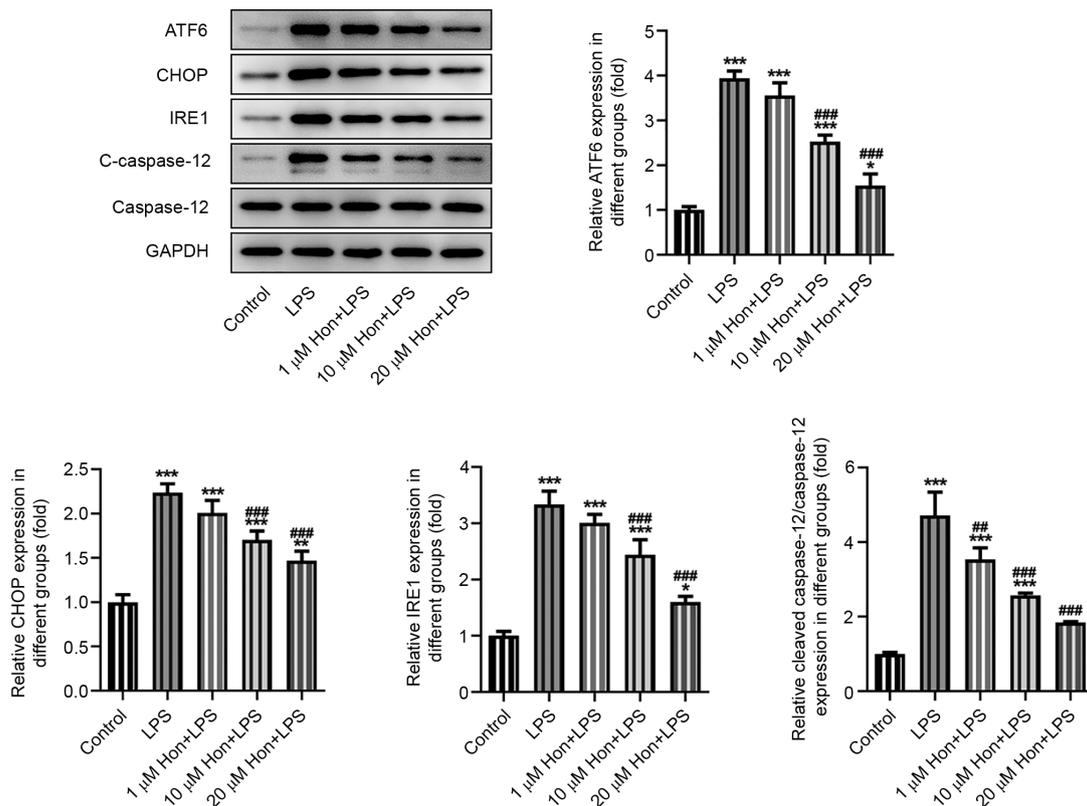


Figure 3. Honokiol inhibits LPS-induced ER stress in bEECs. The expression of ER stress-related proteins in LPS-stimulated bEECs following treatment with different doses of HKL as detected by western blotting. *P<0.05, **P<0.01 and ***P<0.001 vs. control. ##P<0.01, ###P<0.001 vs. LPS. HKL or Hon, honokiol; LPS, lipopolysaccharide; bEECs, bovine endometrial epithelial cells; ATF6, activating transcription factor 6; CHOP, CCAAT-enhancer-binding protein homologous protein; IRE1, inositol-requiring enzyme 1; c-, cleaved.

LPS-induced inflammatory response and apoptosis by mitigating ER stress in bEECs.

Discussion

Endometritis is caused by inflammation in the endometrium, either in the form of acute attacks or in a chronic setting (18). Endometritis is typically caused by bacterial infection or lesions in the uterine cavity (20), which may lead to subfertility or infertility, fetal malformations, premature delivery or miscarriage (2,21,22). Currently available therapeutic strategies for endometritis are limited to antibiotic treatment, intrauterine drug administration, dilation and curettage, which are not favorable either due to the lack of efficacy or are deeply unpleasant for the patient (23-26). Therefore, a milder but more effective treatment option for this condition remain urgently in demand.

HKL is a natural polyphenol that is derived from the traditional Chinese herb *Magnolia officinalis*, also known as Mulan in China and has anti-inflammatory and antioxidant properties (27). It has been previously demonstrated to alleviate oxidative stress and block inflammatory signals to protect against sepsis-induced acute kidney injury (11,28). Chen *et al* (29) showed that HKL modulates the function of the pulmonary microvascular endothelial barrier and ameliorates LPS-induced acute respiratory distress syndrome by the activating sirtuin 3/AMP-activated protein kinase pathway to inhibit angiotensin 2. Although there is a substantial number

of studies on the therapeutic potential of HKL, to the best of our knowledge, its role in endometritis has not been previously reported (12,30). Therefore, the present study was performed to verify this hypothesis.

The *in vitro* model of endometritis in the present study was established through LPS stimulation of bEECs. LPS treatment can induce an inflammatory response that may contribute to the injury and death of cells (31). In addition, since drug treatment require a period of time before it can exert its effect, bEECs were treated with HKL prior to LPS stimulation (32,33). The present study revealed that the viability of bEECs without LPS stimulation was not affected by HKL treatment at any dose, suggesting that HKL exerted no cytotoxic effects on bEECs. Additionally, LPS-stimulated bEECs treated with low to high doses of HKL exhibited increased viability to varying degrees, which provided preliminary proof for the aforementioned hypothesis. Furthermore, this suppressive action of HKL on IL-1β-induced inflammation has been corroborated by a previous study, where the survival and chondrogenesis of human umbilical-cord-derived mesenchymal stem cells was improved by HKL (34). In fact, the anti-inflammatory activity of HKL has been reported in studies on other inflammation-associated diseases. For example, HKL demonstrated protective potential against smoking-induced inflammation, senescence and apoptosis of keratinocytes (35). Liu *et al* (12) also revealed that the levels of proinflammatory markers TNF-α, IL-6 and IL-1β, in addition to those of reactive oxygen species, declined after intraperitoneal injection of HKL into

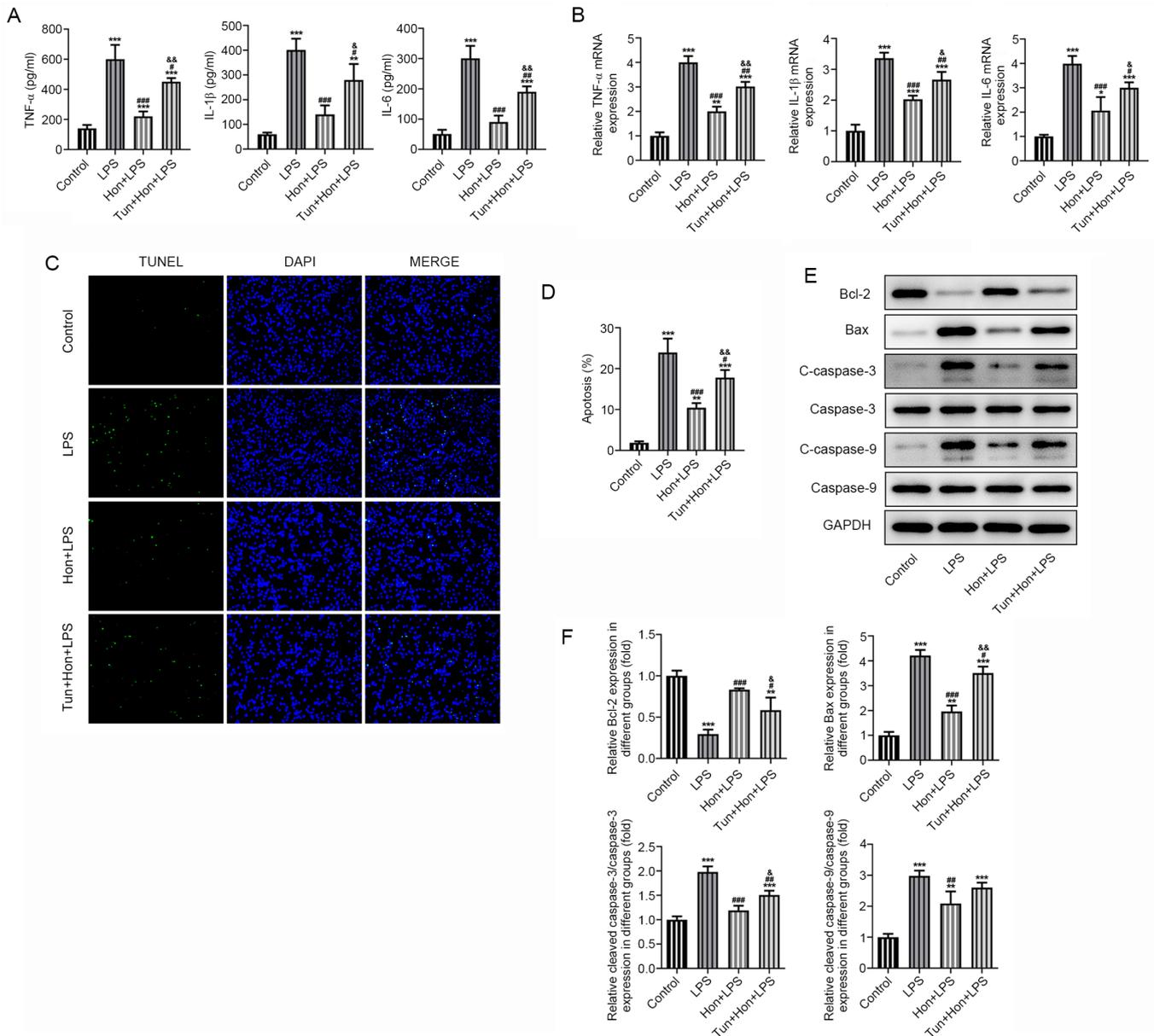


Figure 4. Honokiol mitigates ER stress to inhibit LPS-induced inflammation and apoptosis in bEECs. (A) The expression level of proinflammatory cytokines TNF- α , IL-1 β and IL-6 in LPS-stimulated bEECs treated with 20 μ M HKL in the absence or presence of tunicamycin as detected by ELISA. (B) The mRNA expression of proinflammatory cytokines TNF- α , IL-1 β and IL-6 in LPS-stimulated bEECs treated with HKL in the absence or presence of tunicamycin as detected by reverse transcription-quantitative PCR. (C) The apoptosis level of LPS-stimulated bEECs treated with HKL in the absence or presence of tunicamycin as observed by TUNEL staining, (D) which was quantified. (E) The expression level of apoptosis-related proteins Bcl-2, Bax, cleaved caspase-3 and cleaved caspase-9 in LPS-stimulated bEECs treated with HKL in the absence or presence of tunicamycin as detected by western blotting, (F) which was quantified. * P <0.05, ** P <0.01 and *** P <0.001 vs. control. # P <0.05, ## P <0.01, ### P <0.001 vs. the LPS group. &# P <0.01, && P <0.001 vs. the Hon + LPS group. HKL or Hon, honokiol; LPS, lipopolysaccharide; bEECs, bovine endometrial epithelial cells; tun, tunicamycin.

mice following the induction of carotid artery atherosclerotic lesions. Furthermore, the present results demonstrated that HKL effectively inhibited the expression of proinflammatory cytokines TNF- α , IL-1 β and IL-6, as well as that of proapoptotic proteins, in LPS-stimulated bEECs. By contrast, the expression levels of the anti-apoptotic protein Bcl-2 were markedly upregulated by HKL treatment in a dose-dependent manner. This demonstrated the prominent anti-inflammatory and anti-apoptotic potential of HKL for treating endometritis. In an *in vivo* study in which cognitive disorders and behaviors similar to depression were observed in mice exposed to restraint stress, HKL treatment (10 mg/kg) conferred marked

improvements in these symptoms, whereas inflammation and ER stress levels were both markedly inhibited (36).

ER stress has been previously implicated in the LPS-induced apoptosis of endometrial stromal cells and overexpression of inflammatory cytokines in the goat uterus (8). A close association between ER stress and the activation of thioredoxin-interacting protein/NLR family pyrin domain-containing 3 inflammasome has also been validated in a mouse model of LPS-induced endometritis (9). Therefore, the present study investigated the possible involvement of ER stress in endometritis. LPS enhanced the levels of ER stress-related proteins ATF6, CHOP, IRE1 and cleaved caspase 12

but a notable reduction in the levels of these proteins was observed in bEECs after HKL treatment. Furthermore, LPS and HKL-treated bEECs were treated with the ER stress inducer tunicamycin, which resulted in the reversal of the effects mediated by HKL, revealing the potential regulatory effects of HKL on ER stress in the present endometritis model.

In conclusion, findings of the present study suggested that HKL effectively suppressed ER stress, thereby inhibiting the inflammatory response and apoptosis induced by LPS in bEECs. These findings may further improve our understanding on both the therapeutic potential of HKL and the role of ER stress in the pathogenic process of endometritis. Although the molecular mechanism underlying HKL-mediated protection against endometritis require further study, HKL may be considered as a potentially effective strategy for endometritis treatment.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WC and JW designed the study, drafted and revised the manuscript. SZ and XL analyzed the data and searched the literature. WC, JW and SZ performed the experiments. SZ and XL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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