Piperine Plays an Anti-Inflammatory Role in Staphylococcus aureus Endometritis by Inhibiting Activation of NF-κB and MAPK Pathways in Mice

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Endometritis is commonly caused by pathogenic microorganisms, including Staphylococcus aureus (S. aureus) [3]. S. aureus, which is a Gram-positive bacterium, is a common pathogen that causes purulent infections, such as pneumonia [5] and endometritis [3]. Many reports have shown that S. aureus induces inflammatory injury through TLRs and their downstream pathways, such as the NF-κB and MAPK pathways [6, 7].

1. Introduction

Endometritis is a major disease in the postpartum period, and it affects both human and animal health [1, 2]. Endometritis can be caused by a variety of pathogens, including Staphylococcus aureus (S. aureus) [3] and Escherichia coli [4]. S. aureus, which is a Gram-positive bacterium, is a common pathogen that causes purulent infections, such as pneumonia [5] and endometritis [3]. Many reports have shown that S. aureus induces inflammatory injury through TLRs and their downstream pathways, such as the NF-κB and MAPK pathways [6, 7].

TLR-2 and TLR-4, which are members of the TLR family, are pivotal receptor proteins on the surface of cells mediating natural anti-infection immunity [8]. TLR-2 usually identifies the antigen on the surface of S. aureus [9], and some recent studies have shown that TLR-4 also plays a similar role [10]. NF-κB and MAPKs are important molecules that should receive the signal transduced from the transformation of membrane receptors and, in turn, transmit it into the cell nucleus [11, 12]. TLR-2 and TLR-4 activation promotes the recruitment of adaptor proteins to activate NF-κB [13], and some studies have indicated that MAPKs are also involved [14]. Activated NF-κB and MAPKs stimulate the S. aureus-infected cells to produce a variety of inflammatory factors, such as the proinflammatory factors TNF-α, IL-1β, and IL-6 and the anti-inflammatory factor IL-10 [11].

Piperine, which is the major plant alkaloid from black pepper (Piper nigrum) and long pepper (Piper longum), has been reported to enhance bioavailability [15]. Previous studies have shown that piperine has many biological functions, including analgesic [16], anticonvulsant [17], antitumour [18], and anti-inflammatory [19] properties. The inflammatory reaction, a biological defence and repair mechanism of the
innate immune system, is used to prevent harmful irritants, such as pathogens, from damaging cells and tissues. In the
present study, an S. aureus endometritis model was success-
fully established in mice to explore the potential anti-
inflammatory mechanism of piperine, which was not previ-
ously known.

2. Materials and Methods

2.1. Reagents. Piperine (purity: >98%, Figure 1) was pur-
chased from the National Institute for the Control of Phar-
rnaceutical and Biological Products (Beijing, China). TRIzol
was obtained from Invitrogen. IL-1β, IL-6, IL-10, and TNF-α
ELISA kits were acquired from BioLegend (Camino Santa Fe,
CA, USA). All antibodies were purchased from Cell Signaling
Technology (Beverly, MA, USA). S. aureus (ATCC 35556)
was purchased from the American Type Culture Collection
(ATCC, USA).

2.2. Animals and Experimental Groups. A total of 60 female
BALB/c mice were used in this study. All of these mice
were acquired from the Experimental Animal Center of
Wuhan University (Wuhan, China). All experiments followed
the guidelines for the care and use of laboratory animals
published by the US National Institutes of Health. This
study was approved by the Huazhong Agricultural University
Animal Care and Use Committee. All mice were maintained
on a 12 h light/dark cycle and cafeteria feeding.

Piperine was dissolved in 5 mL of tris buffered saline
(TBS) at concentrations corresponding to 25, 50, and 100 mg/
kg, based on the weight of the mice. After 24 h of S. aureus
infection in the uterus, the piperine solution was injected
intraperitoneally three times every 6 h. All mice were stochas-
tically split into 4 groups as follows: (1) control group (CG),
consisting of healthy mice that received no treatment; (2)
S. aureus group (S. aureus), consisting of mice that received
an injection of 100 μL S. aureus in each side of the uterus
horn and no drug treatment; (3) piperine treatment groups
(PTGs), in which the S. aureus-infected mice were intraperi-
toneally injected with 25, 50, or 100 mg/kg of piperine; and
(4) DEX group (DEX), in which the S. aureus-infected mice
were cured by DEX. The mice were euthanized with sodium
pentobarbital, and the uterus tissues were collected and
stored at −80°C until analysis.

2.3. Histological Assays. The uterus tissues were gathered and
fixed in 10% neutral buffered formalin. The uterus samples
were embedded in paraffin, sliced, and stained with H&E
reagent. After staining, the pathologic tissue sections were
examined with a light microscope (Olympus, Japan).

2.4. Enzyme-Linked Immunosorbent Assays. The effects of
piperine on the expression levels of S. aureus-induced inflam-
atory factors were examined in the uterus tissues. The
uterus tissues were weighed and homogenized in phos-
phate buffered saline (PBS) on ice and then centrifuged at
12,000 rpm for 15 min at 4°C, and the supernatants were
collected. The levels of TNF-α, IL-1β, IL-6, and IL-10 were
detected by ELISA kits in the supernatants of uterus tissues,
according to the manufacturer’s directions. Each sample assay
was repeated three times. The absorbance was read at 450 nm
with a microplate reader (Thermo Scientific Multiskan MK3,
USA).

2.5. Quantitative Real-Time Polymerase Chain Reaction (q-
PCR) Assays. The total RNA was extracted from the uterus
tissue samples of each group using TRIzol reagent according
to the manufacturer’s instructions (Invitrogen, China).
The concentration and purity were determined spectrophotomet-nically at 260 and 280 nm. First-strand cDNA was synthesized
using oligo. primers and Superscript II reverse transcriptase,
according to the manufacturer’s instructions (Invitrogen,
USA). The synthesized cDNA was diluted five times with
sterile water and stored at −80°C. The Primer Premier soft-
ware (Premier Biosoft International, USA) was used to design
specific primers for TNF-α, IL-1β, IL-6, IL-10, TLR2, TLR4,
and GAPDH based on known sequences (Table 1). The qPCR
was performed with an ABI PRISM 7500 Detection System
(Applied Biosystems, USA). Reactions were performed in a
20 μL reaction mixture containing 10 μL of 2x SYBR Green I
PCR Master Mix (TaKaRa, China), 1 μL of diluted cDNA, 1 μL
of each primer (10 μM), 0.8 μL of 50x ROX Reference Dye II,
and 6.2 μL of PCR-grade water. The qPCR conditions were as
follows: 95°C for 10 min, then 40 cycles of 95°C for 15 s, and
60°C for 60 s and 72°C for 60 s. The results (fold change) were
expressed as 2^(-ΔΔCt). GAPDH was the reference gene. Each
sample assay was repeated three times.

2.6. Western Blot Assays. The total protein was extracted from
100 mg of uterus tissues according to the manufacturer’s rec-
ommended protocol (Invitrogen, Beijing, China). The BCA
Protein Assay Kit was used to determine the protein concen-
trations. Samples with equal amounts of protein (50 μg) were
fractionated with 10% SDS polyacrylamide gels, transferred
to polyvinylidene difluoride membranes, and blocked in 5% skim
milk in TBST for 2 h. Then, the membranes were incu-
bated with 1:1000 dilutions (v/v) of the primary antibodies at
4°C for 12 h. They were then incubated for 2 h with secondary
antibodies diluted to 1: 4000 (v/v). Densitometric values
of immunoblot signals were developed with the ECL Plus
Western Blotting Detection System (ImageQuant LAS 4000 mini, USA). Each sample assay was repeated three times. β-actin was used as a loading control.

2.7. Statistical Assays. Statistical analyses of the result data were performed using the SPSS software (ver. 15 for Windows; SPSS Inc., Chicago, IL, USA). All values are expressed as the mean ± SD. Significance was determined by a one-way ANOVA. A p value of < 0.05 was considered statistically significant.

3. Results

3.1. Histopathological Changes. The inflammation injury could be directly seen with histopathological observation. The results are shown in Figure 2. No other uterine pathological changes were found in the CG (Figure 2(a)). In contrast to the CG, the S. aureus-treated samples showed injury (Figure 2(b)). The results showed that many of the epithelial cells were destroyed and that the hyperemia-related uterus tissues were infiltrated with a host of inflammatory cells, such as neutrophils and macrophages. These histopathological changes were ameliorated following treatment with piperine at doses of 25, 50, and 100 mg/kg (Figures 2(d)–2(f)).

3.2. Effects of Piperine on S. aureus-Induced Inflammatory Cytokines. The ELISA and qPCR methods were used to analyze the expression of TNF-α, IL-1β, IL-6, and IL-10. The results are shown in Figure 3. The results showed that S. aureus apparently increased the expression of TNF-α, IL-1β, IL-6, and IL-10 compared with that in the CG. Compared with their expression in the S. aureus group, the expression of TNF-α, IL-1β, and IL-6 in the PTGs (25, 50, or 100 mg/kg) was clearly reduced in a dose-dependent manner (Figure 3(a)). However, the expression of IL-10 in the PTGs (25, 50, or 100 mg/kg) increased more than that in the S. aureus group in a dose-dependent manner (Figure 3(a)). The results showed that piperine can reduce the expression of proinflammatory factors (TNF-α, IL-1β, and IL-6), but it only improves the expression of anti-inflammatory cytokine IL-10. Furthermore, this effect became increasingly evident with an increasing piperine dosage (25, 50, or 100 mg/kg). The respective mRNA expression was consistent with the protein expression (Figure 3(b)).

3.3. Effects of Piperine on Expression of TLR-2 and TLR-4. TLR-2 and TLR-4 play key roles in S. aureus-mediated inflammatory responses [14]. The recognition of S. aureus by TLR-2 and TLR-4 can directly impact the activation of NF-κB and MAPKs. The results showed that the mRNA levels of TLR-2 and TLR-4 increased significantly upon S. aureus infection. The S. aureus-induced increase was significantly (p < 0.05) suppressed by piperine in a dose-dependent manner (25, 50, or 100 mg/kg). The results are shown in Figure 4.

3.4. Effects of Piperine on the NF-κB Pathway in S. aureus-Induced Endometritis. The NF-κB signalling pathway is important for the inflammatory reaction of S. aureus infection [11]. To determine the effects of piperine, the NF-κB activation was analyzed by western blot. The results showed that the phosphorylation of NF-κB p65 and I-κBα was significantly higher with S. aureus-infected mice than in the CG. The phosphorylated protein expression of NF-κB p65 and I-κBα was significantly inhibited by piperine treatment compared with their expression in S. aureus-infected mice that were not treated with piperine. With increasing concentrations of piperine (25, 50, and 100 mg/kg), the inhibition became more obvious, as shown in Figure 5.

3.5. Effects of Piperine on S. aureus-Induced MAPK Signalling Pathways. MAPKs, which are activated by S. aureus, play significant roles in the development of endometrial inflammation [11]. To further determine the anti-inflammatory mechanism of piperine, the MAPKs (p38, ERK, and JNK) were examined by western blot, as shown in Figure 6. The phosphorylation of p38, ERK, and JNK was higher in S. aureus-stimulated uterus tissues. In the PTGs, the phosphorylation of p38, ERK, and JNK was significantly reduced compared with that in the S. aureus group. The results

Table 1: Sequence of primers used for qPCR assay.

| Gene       | Accession number | Primers                       | Product |
|------------|------------------|-------------------------------|---------|
| IL-1β      | NM_008361.3      | Sense: 5'-AGGCTCGAGAATGCAA-3' | 264 bp  |
|            |                  | Anti-sense: 5'-AGGCGATTAACAGGC-3' |         |
| TNF-α      | NM_013693.2      | Sense: 5'-ACTTGGTTGGTACCC-3' | 198 bp  |
|            |                  | Anti-sense: 5'-ACTTGGTTGGTACCC-3' |         |
| IL-6       | NM_031168.1      | Sense: 5'-CTGGCTTTTGTTTCTGT-3' | 180 bp  |
|            |                  | Anti-sense: 5'-CTGGCTTTTGTTTCTGT-3' |         |
| TLR-2      | NM_011905.3      | Sense: 5'-TTTGCCTCCTGGACTCC-3' | 267 bp  |
|            |                  | Anti-sense: 5'-TTTGCCTCCTGGACTCC-3' |         |
| TLR-4      | NM_021297.2      | Sense: 5'-AGTGGTTGTGAACCAGT-3' | 170 bp  |
|            |                  | Anti-sense: 5'-AGTGGTTGTGAACCAGT-3' |         |
| GAPDH      | NM_008084.2      | Sense: 5'-GGGTCGTTGAATGCAACA-3' | 195 bp  |

3.4. Effects of Piperine on the NF-κB Pathway in S. aureus-Induced Endometritis. The NF-κB signalling pathway is important for the inflammatory reaction of S. aureus infection [11]. To determine the effects of piperine, the NF-κB activation was analyzed by western blot. The results showed that the phosphorylation of NF-κB p65 and I-κBα was significantly higher with S. aureus-infected mice than in the CG. The phosphorylated protein expression of NF-κB p65 and I-κBα was significantly inhibited by piperine treatment compared with their expression in S. aureus-infected mice that were not treated with piperine. With increasing concentrations of piperine (25, 50, and 100 mg/kg), the inhibition became more obvious, as shown in Figure 5.
Figure 2: Histopathological changes of uterus tissues (H&E, ×200). (a) In the control group (CG), the mice received no treatment. (b) In the S. aureus group (S. aureus), the mouse model of S. aureus endometritis received no drug treatment. (c) In the DEX group (DEX), mice with S. aureus endometritis were treated with 5 mg/kg of dexamethasone. (d–f) In the piperine treatment groups (PTGs), mice with S. aureus endometritis were given 25 mg/kg, 50 mg/kg, or 100 mg/kg of piperine.

4. Discussion

Endometritis is a reproductive inflammatory disease in the postpartum period [20]. Many reports have shown that S. aureus can induce uterine inflammation [3, 21]. In the present study, a mouse model of S. aureus endometritis was successfully established. *S. aureus* injury was relieved by piperine, in agreement with the results of a previous study [22]. Histopathology changes showed that *S. aureus* induced serious injury to the uterus of mice, including nematode damage to the uterine structure and a decline in productivity and fertility [23]. *S. aureus* infection increased the secretion of inflammatory cytokines, which is consistent with the findings of a previous study [24]. Piperine significantly inhibited the *S. aureus*-induced excessive secretion of proinflammatory factors (TNF-α, IL-1β, and IL-6) but increased the secretion...
Figure 3: Expression of inflammatory cytokines. (a) The protein production levels of TNF-α, IL-1β, IL-6, and IL-10 in uterus tissues. (b) The mRNA levels of TNF-α, IL-1β, IL-6, and IL-10 in uterus tissues are shown. CG: control group. *S. aureus* group. The 25, 50, and 100 indicate the piperine treatment groups, which were given piperine at 25 mg/kg, 50 mg/kg, and 100 mg/kg per animal. DEX: DEX group, treated with 5 mg/kg. The data are represented as the mean ± SD. *p* < 0.05 represents statistical significance compared with the CG and *#p* < 0.05 represents statistical significance compared with the *S. aureus* group.
Figure 4: Effects of piperine on TLR-2 and TLR-4. The mRNA levels of TLR-2 and TLR-4 in uterus tissues. qPCR was performed to detect the TLR-2 and TLR-4 mRNA levels. GAPDH was used as a control. CG: control group, the mice without any treatment. S. aureus: the mouse model of S. aureus endometritis without any drug treatment. 25, 50, and 100: piperine treatment groups: the S. aureus endometritis mice were given piperine at 25 mg/kg, 50 mg/kg, and 100 mg/kg. DEX: the S. aureus endometritis mice were treated with dexamethasone at 5 mg/kg. The data are represented as the mean ± SD. *p < 0.05 is significantly different from the CG; #p < 0.05 is significantly different from the S. aureus group.

Figure 5: Effects of piperine on NF-κB pathway activation. (a) The expression of NF-κB p65 and I-κBα is shown. (b) The ratio of NF-κB p65 or I-κBα and β-actin. CG: control group, the mice without any treatment. S. aureus: the mouse model of S. aureus endometritis without any drug treatment. 25, 50, and 100: piperine treatment groups: the S. aureus endometritis mice were given piperine at 25 mg/kg, 50 mg/kg, and 100 mg/kg. DEX: the S. aureus endometritis mice were treated with dexamethasone at 5 mg/kg. β-actin was used as a control. Values are presented as the means ± SD. *p < 0.05 significantly compared with the CG; #p < 0.05 significantly compared with the S. aureus.
Figure 6: Effect of piperine on MAPK pathways activation. (a) The expression of p38, ERK, and JNK is shown. (b) The ratios of p38 or ERK or JNK with β-actin are shown. CG: control group, the mice without any treatment. S. aureus: the mouse model of S. aureus endometritis without any drug treatment. 25, 50, and 100: piperine treatment groups: the S. aureus endometritis mice were given piperine at 25 mg/kg, 50 mg/kg, and 100 mg/kg. DEX: the S. aureus endometritis mice were treated with dexamethasone at 5 mg/kg. β-actin was used as a control. *p < 0.05 represents statistical significance compared with the CG and #p < 0.05 represents statistical significance compared with the S. aureus group.
of an anti-inflammatory factor (IL-10). Additionally, these inflammatory responses were controlled by piperine in a dose-dependent manner.

TNF-α and IL-1β, which are typical primary cytokines, play a role in initiating the inflammatory response [25]. TNF-α induces the infiltration and activation of neutrophils and enhances the expression of cellular adhesion molecules, but it impairs endothelial cells and aggravates the cascade of other inflammatory mediators [26, 27]. The expression of TNF-α was reduced after piperine treatment, which indicates that piperine could alleviate inflammation by inhibiting the effects of neutrophils on the cells in the endothelial monolayer [28]. IL-1β, which is a major catalytic factor, is key in the regulation of host immune responses against *S. aureus* [29,30]. The decrease in IL-1β expression proved that piperine could resist inflammation. IL-6 plays an essential role in neutrophilic inflammation [31]. IL-6, which is promptly and transiently produced in infections and tissue injury responses, contributes to the host defence through the stimulation of acute-phase responses, hematopoiesis, and immune reactions [32]. The decrease in IL-6 expression indicated that piperine had an anti-inflammatory effect. In contrast, IL-10 may be necessary to reduce collateral injury due to inflammatory responses [33]. IL-10, which is a multifunctional cytokine, plays a pivotal role in suppressing the production of proinflammatory mediators [34]. The increase in the IL-10 expression level meant that piperine could play an anti-inflammatory role. TNF-α, IL-1β, IL-6, and IL-10 have been implicated in the initiation and regulation of the immune response and inflammation [14,35,36]. In summary, piperine showed anti-inflammatory effects by decreasing the overexpression of proinflammatory factors (TNF-α, IL-1β, and IL-6) and increasing the expression of an anti-inflammatory factor (IL-10). Additionally, the results of the present study are in agreement with those of previous studies [37, 38].

*S. aureus* is identified mainly through TLR-2 [24], but some studies have confirmed that TLR-4 also plays an important role in the process of *S. aureus*-induced inflammation [10]. The stimulation of TLR-2 and TLR-4 can lead to the production of different cytokines [14]. TLR-2 and TLR-4 activated downstream pathways to improve the expression of inflammatory factors (TNF-α, IL-1β, IL-6, and IL-10). Many studies have shown that the excessive expression of TLR-2 and TLR-4 promoted an active inflammatory reaction [14,39]; the reduction of TLR-2 and TLR-4 expression in this study confirmed that piperine could inhibit inflammation.

To further probe the anti-inflammatory mechanism of piperine, the expression of NF-κB (NF-κB p65 and I-κBα) and MAPKs (p38, ERK, and JNK) was examined by western blot. NF-κB is a ubiquitous heterodimeric transcription factor that exists in an inactive form in the cytoplasm, bound to the inhibitory proteins referred to as I-κB [40]. The NF-κB pathway has been shown to be involved in numerous inflammatory diseases [2,11]. The suppression of NF-κB p65 and I-κBα showed the anti-inflammatory effect of piperine. MAPKs are part of another pathway in the regulation of inflammatory responses and include the proteins p38, ERK, and JNK [12]. In the present study, the phosphorylation of p38, ERK, and JNK was significantly decreased by piperine. This showed that piperine played an anti-inflammation role by inhibiting the expression of p38, ERK, and JNK, and these results were consistent with those from previous studies [41,42].

5. Conclusion

In conclusion, piperine showed an anti-inflammatory effect in *S. aureus*-induced endometritis. Piperine inhibited the expression of TLR-2 and TLR-4 and the activation of the NF-κB and MAPKs pathways, thereby preventing the excessive secretion of proinflammatory factors (TNF-α, IL-1β, and IL-6) and increasing the secretion of an anti-inflammatory factor (IL-10). After further research, piperine may be an effective drug for the clinical therapy of *S. aureus* endometritis and other infections.

Competing Interests

The authors declare that they have no competing interests.

Authors’ Contributions

Wen-jun Zhai and Zhen-biao Zhang made equal contributions to this paper.

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