Am J Transl Res 2022;14(11):8332-8342
www.ajtr.org /ISSN:1943-8141/AJTR0143666

Original Article

Polysaccharide derived from *Inonotus obliquus* inhibits lipopolysaccharide-induced acute endometritis in mice

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Received April 20, 2022; Accepted November 8, 2022; Epub November 15, 2022; Published November 30, 2022

Abstract: Objective: Endometritis bacterial pathogenic condition that affects both humans and animals develops in the inner lining of the uterus. *Inonotus obliquus* polysaccharide (IOP), an active cocktail of *Inonotus obliquus*, has been shown to have a relatively wide range of biological activities and can play a role in various diseases. However, from the currently reported article, there is no information about the anti-inflammatory effect of IPO in the symptoms of lipopolysaccharide (LPS)-induced endometritis. Therefore, this study carefully observed the phenomenon of IOP on the symptoms of endometritis induced by LPS in mice, elucidated the protective mechanism of IOP on the body, and clarified the potential mechanism of IOP. Methods: A total of 72 BALB/c female experimental mice were divided into several groups for comparison. They were the blank control group, the LPS group, the LPS+ IOP group (the effect of IOP dose on mice was also explored, divided into low, medium, and high) and LPS+ amoxicillin group. All groups except control group were infused with LPS into the uterus. The mice of LPS+ IOP groups and LPS+ amoxicillin group were orally administered with IOP or amoxicillin after LPS challenge for 3 hours. Histopathology and myeloperoxidase (MPO) activity were used to detect uterine tissue injury, and cytokine levels were used to measure uterine inflammation. The expression of toll-like receptor 4/nuclear factor-kappa B (TLR4/NF-κB)-related proteins in the inflammatory signaling pathway was observed. Results: Pathological and MPO activity analyses revealed that IOP relieved LPS-induced uterine tissue injury. Quantitative reverse transcription-polymerase chain reaction was used to detect and quantitatively study the RNA information of mouse cells, which had high accuracy and sensitivity. From the test results, IOP does effectively control the release of pro-inflammatory cytokines such as interleukin-6 (IL-6), IL-1β, IL-8 and tumor necrosis factor-α (TNF-α), avoiding the body's immune response. Analysis of uterine tissue cell components also confirmed that the expression level of inflammatory mediator-induced nitric oxide synthase (iNOS) was also greatly reduced. Analysis of western blotting results of cell synthesis showed that IOP mainly inhibited the protein expression of TLR4 and myeloid differentiation factor 88 in the body. Conclusion: This study proved that the mechanism of action of IOP is to inhibit the TLR4/NF-κB signaling pathway to reduce the release of pro-inflammatory cytokines from body cells, thereby alleviating the symptoms of endometritis induced by LPS. Thus, IOP may act as an effective drug in preventing and curing LPS-induced endometritis.

Keywords: *Inonotus obliquus* polysaccharide, lipopolysaccharide, endometritis, TLR4, anti-inflammatory

Introduction

Endometritis is a reproductive disorder that affects humans and animals and can reduce their fertility or even cause infertility [1-4]. Some common bacterial species that cause endometritis include *Corynebacterium*, *Enterococcus*, *Escherichia coli*, and *Staphylococcus aureus* [5, 6]. Lipopolysaccharides (LPS) are observed in the gram-negative bacterial cell wall (such as *E. coli*) and are considered immune stimulators. Studies on animals have also reported that LPS can effectively relieve the symptoms of acute lung disease and reduce the onset of acute endometritis and pulpitis [7-9].

The uterine environment of mammals is relatively sterile secondary to innate immunity, which resists the infection of pathogenic micro-

organisms. Its immune response system is the body's first line of defense against pathogen invasion and the primary cause of the inflammatory response. Inflammation is caused by changes in immune response, including the cellular response, involving macrophages, granulocytes, and dendritic cells, and is mainly caused by pattern recognition receptors (PRRs) that are expressed on the surface of innate immune cells. Additionally, PRRs regulate innate immunity by identifying evolutionarily conserved pathogen-associated molecular patterns [10]. Toll-like receptor 4 (TLR4) is a member of the TLR family (a type of PRR) and can recognize a wide range of ligands, especially the LPS of bacteria [11, 12]. After ligand recognition, several downstream signal pathways can be activated. But the main thing is to activate the MyD88-dependent myeloid differentiation factor 88 (MyD88) signaling pathway. Because MyD88 can make nuclear transcription factor (NF-κB), induce the release of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6), which will aggravate the severity of inflammation in the body [13, 14], and in severe cases, it will become a long-term inflammatory disease such as rheumatoid arthritis [15] and pneumonia [16]. Thus, inhibiting the TLR4/NF-κB signaling pathway may help reduce LPS-induced endometritis.

Clindamycin and doxycycline are some of the medicines frequently prescribed to treat endometrial inflammation [17]. The treatment of bacterial infections has also included the use of several other antibiotics with improved bioavailability or antibiotic combinations. However, the emergence of bacterial resistance is a common consequence of the continued use of these drugs [18]. Therefore, it is vital to explore new methods of endometritis treatment. Currently, natural products with high potency and low toxicity are being increasingly used to cure bacterial endometritis [8, 19-21]. *Inonotus obliquus* is an esculent medicinal fungus that flourishes throughout Asia and Europe under frosty conditions. This is a well-known folk treatment in Russia, which can effectively improve the effects of symptoms of tumors and gastric ulcers. In addition, the drug has also gained wide acceptance in Europe. It is considered by the medical community to be an effective means of preventing and treating cancer, alleviating cerebrovascular diseases and diabetes [22-24]. Its constituent polysaccharides exhibit numerous biological characteristics, for instance, the anti-inflammatory, anti-oxidation, and antiviral properties [25-27]. The antioxidant activity of *Inonotus obliquus* polysaccharides (IOPs) inhibits diethyldithiocarbamate, which causes chronic pancreatitis in mice [28]. The alkali-solubility of IOP promotes lymphocyte transformation and proliferation, enhances the phagocytic function of macrophages, improves the immune functioning of the body, and assists in cancer treatment [29]. Additionally, IOP regulates matrix metalloproteinase expression and inhibits tumor cell metastasis [30]. Nevertheless, its role in endometritis remains elusive. This study aimed to investigate the mechanism underlying the protective effect of IOP in LPS-induced endometritis in mice.

Materials and methods

Materials

Inonotus obliquus used in the experiment was provided by the Veterinary Laboratory of RUDN University. Divided into standard monosaccharides and uronic acids, there are rhamnose (Rham), mannose (Man), glucuronic acid (Glu A), ribose, galactose (Gal), galacturonic acid (Gal A), xylose (Xyl), glucose (Glu), fucose (Fuc) and arabinose (Ara). In addition, phosphate buffered solution (PBS) was purchased from Beijing Labgic Technology Co., Ltd.; LPS was obtained from Sigma-Aldrich, USA; amoxicillin was purchased from Renhe Pharma Co. (Shenzhen, China). Antibodies against TLR4 (D220102, 1:2000) and MyD88 (D121009, 1:2000) were purchased from Sangon Biotech in Shanghai, China. Antibodies against the phosphorylated nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor, alpha (p-IκBα) (4812, 1:2000), p-p65 (3226, 1:2000), IκBα (2859, 1:2000), p65 (8242, 1:2000), and β-actin (4970, 1:5000) were purchased from Cell Signaling Technology, USA.

Extraction and purification of IOP

IOP is traditionally prepared, first advanced by heat treatment, and then purified by centrifugation [31]. The shell of *Inonotus obliquus* was removed, pulverized, extracted with hot water,

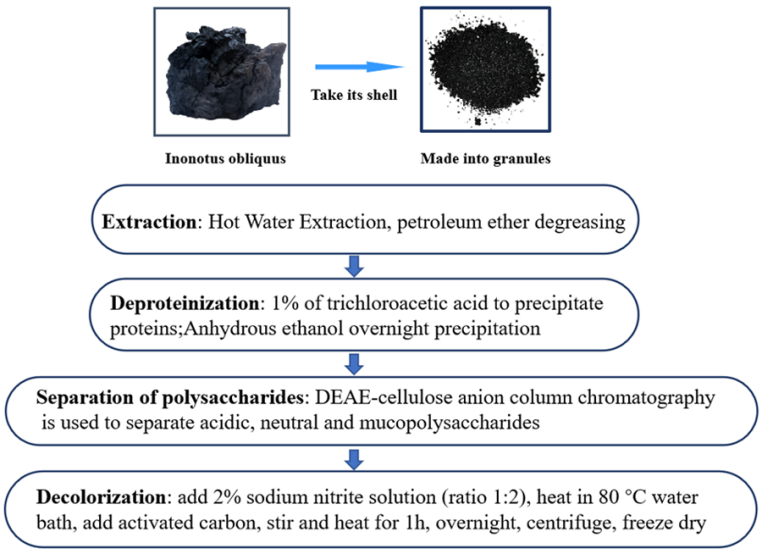

Figure 1. The extraction and purification of *Inonotus obliquus* polysaccharides (IOPs).

and petroleum degreasing ether. After oil removal, the residue was dried at a low temperature. The protein precipitates by adding trichloroacetic acid at a concentration of 1%, and the filtrate is concentrated into a liquid extract after centrifugation. Then wash the standing pellet with absolute ethanol, store the solution at an ambient temperature of 0°C overnight, and take it out and treat it with a high-speed centrifuge the next day to finally obtain a crude polysaccharide precipitate. The acidic and neutral mucopolysaccharides were separated using diethylaminoethyl cellulose anionic column chromatography. Further, 2% sodium nitrite solution (1:2) was added to the water bath at 80°C, and activated carbon was added to stir for 1 h. As shown in **Figure 1**, refined polysaccharides can be obtained by centrifugation after the solution is left to stand overnight, and the pellet is dried.

Monosaccharide and uronic acid compositional analysis of IOP

High performance liquid chromatography is currently the most effective method for the determination of carbohydrates contained in chemical components, so this paper uses this method to analyze the monosaccharides and uronic acids of IPO. 3.41 milligrams of IOP were carefully added into the ampoule, followed by pouring 0.5 mL of 4 M trifluoroacetic acid. Nitrogenwas introduced into the ampoule for 5 minutes, and then the ampoule was sealed and placed at an ambient temperature of 121°C for 2 h. After removal, trifluoroacetic acid was dried with nitrogen. Finally, 0.5 mL of 1-phenyl-3-methyl-5-pyrazolinone and 0.5 mL of 0.3 M NaOH were added. In this process, it was necessary to keep the solution temperature at 70°C, open it by water bath insulation, and take it out for cooling after 1 hour. Then filled 0.5 mL of 0.3 M HCl. DNA extraction by chloroform required three times, and the supernatant was centrifuged after the third extraction. 20 μL was packed into a reagent bottle. It was passed through a 0.22-nm membrane before injection. The experimental conclusions and standard curves obtained by high performance liquid chromatography for the analysis of standard monosaccharides and uronic acid are shown in **Table 1**.

Experimental procedure

Seventy-two BALB/c female mice (6-8 weeks old) were purchased from Dossy Experimental Animals Co. (Chengdu, China) and these mice were allowed to live at an ambient temperature of 25±3°C and provided with living conditions that were replaced by light and dark cycles every 12 hours, maintaining an ambient relative humidity of 75±5% and providing the mice with the food and water they needed. This research protocol was approved by the Office of Animal Care of Chengdu Normal University (CDNU-2021092614M). It is worth mentioning that this experiment was conducted in accordance with the guidelines for the care of laboratory animals formulated by the state.

All mice were divided into six groups for comparison, and the number of mice in each group was 12. The six groups were: blank control group, LPS experimental group, LPS+ high-dose IOP, LPS+ medium-dose IOP, and LPS and amoxicillin experimental group. The LPS-induced endometritis model was constructed referring to the instructions reported in previous studies [8, 32]. Use of 1% pentobarbital

Table 1. The regression equation and R^2 of glycuronic acid and monosaccharides

Glycuronic acid and monosaccharides	Regression equation	R^2
man	y=0.0002x-0.0069	1
rham	y=0.00010x-0.06978	0.99995
ribose	y=0.00006x-0.01788	0.99983
glu A	y=0.0001x+0.0027	1
gal A	y=0.0001x-0.0124	0.9999
gal	y=0.00033x-0.01613	0.99929
glu	y=0.0001x-0.0113	1
xyl	y=0.0005x-0.0073	0.9997
ara	y=0.0008x-0.0095	0.9999
fuc	y=0.0003x-0.0032	1

Man: Mannose; Rham: Rhamnose; Glu A: Glucuronic Acid; Gal A: Galacturonic Acid; Gal: Galactose; Glu: Glucose; Xyl: Xylose; Ara: Arabinose; Fuc: Fucose.

sodium (80 mg/kg) [33] to anesthetize mice is most effective. It was injected with 20 μL LPS on each side of the uterus, and the control received an equal volume of PBS. Three hours following the LPS injection, mice in the LPS+ IOP group (low-dose, medium-dose, high-dose) were orally administered with IOP (50 mg/kg, 100 mg/kg, and 150 mg/kg, respectively [34]), and the LPS+ amoxicillin group was orally administered with amoxicillin (100 mg/kg). Nine hours later, the mice were killed by rapid dislocation of the neck, with every attempt being taken to minimize suffering. The uterine tissue was collected and preserved at -80°C.

Cell culture and viability detection

The mouse endometrial epithelial cells (mEECs) were isolated and cultured as previously mentioned [35]. The uterine tissue was shredded, washed using Hank's solution, and then digested with 0.25% trypsin-ethylene diamine tetraacetic acid digestive solution at 37°C for 30 min. The homogenized cells were centrifuged and filtered with a 20-μm nylon membrane. The cells were further cultured in Dulbecco's Modified Eagle Medium/F-12 medium in an incubator containing 5% CO_2 at 37°C. CCK-8 is used to quickly assay highly sensitive cell activity. Cells were transferred into a 96-well plate, and different concentrations of IOP (5, 10, and 15 μg/mL [34]) were added, after which they were placed in the incubator for culturing for 24 h (37°C, 5% CO_2). Then 10 μL of CCK-8 solution was added dropwise to each well and incubated for 1 to 4 hours. Finally, the absorbance at 450 nm was tested with a microplate reader.

Histopathological analysis

Uterine tissue cells were fixed with paraformaldehyde, and the cells were trimmed and dehydrated. The cells were embedded in paraffin when finished, and 5-μm thick slices were stained with Hematoxylin & Eosin and observed under a microscope (Nikon, Eclipse Ci-L, Japan).

Myeloperoxidase (MPO) detection

Enzyme-linked immunosorbent assay (ELISA) is currently the most commonly used labeling and analysis immunoassay. ELISA was used to detect the effect of IOP on LPS-induced MPO activity.

Quantitative polymerase chain reaction (PCR)

Trizol reagents can extract the required RNA information directly from tissue cells, even in sample lysis solutions, Trizol can guarantee the integrity of advanced RNA. In this article, TRIzol reagent was chosen to extract RNA from uterine tissue cells. The RNA is then reverse-transcribed into complementary DNA, known as cDNA. First, soak the reaction mixture at 65°C for 5 minutes, then take out the ice for 2 minutes, and finally, 4 μL 5 × RT Buffer, 0.5 μL Thermo Scientific RiboLock RNase Inhibitor, and 1 μL Maxima Reverse Transcriptase (Thermo Fisher Scientific, USA) were added. The PCR tube was placed at 25°C for 10 min, incubated at 50°C for 30 min, and denaturized at 85°C for 5 min to obtain cDNA. Two microliters of cDNA were added into a 0.2-mL PCR tube and mixed with 10 μL of 2 × SG Fast qPCR Master Mix (High Rox, BBI, ABI). 0.4 μL forward primers, 0.4 μL reverse primers, and 3 μL ddH_2O were added and analyzed in the StepOne™ Real-Time PCR System. **Table 2** lists the primer sequences used in this study that were designed using Primer Premier v5.0 software and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key enzyme involved in glycolysis. In addition, the expression content of the target gene was quantified using the $2^{-\Delta\Delta Ct}$ method.

Western blot

The quantitative measurement of protein content is BCA (bicinchoninic acid) method, which

Table 2. Primers used in a series of reactions with reverse transcription polymerase

Name	Primer sequence	Product size (bp)
TNF-α	Forward: TGTCTCAGCCTCTTCTCATTCC	152
	Reverse: TTTGTGAGTGTGAGGGTCTGG	
IL-6	Forward: TCTTGGGACTGATGCTGGTG	132
	Reverse: CATGTGTAATTAAGCCTCCGACT	
IL-8	Forward: TAGGCATCTTCGTCCGTCC	212
	Reverse: GCCAACAGTAGCCTTCACCC	
IL-1β	Forward: GTAATGAAAGACGGCACACCC	181
	Reverse: CAGGCTTGTGCTCTGCTTGTG	
iNOS	Forward: GAGCGAGTTGTGGATTGTC	207
	Reverse: CTGCCTATCCGTCTCGTC	
GADPH	Forward: GGTTGTCTCCTGCGACTTCA	183
	Reverse: TGGTCCAGGGTTTCTTACTCC	

TNF-α: Tumor Necrosis Factor-Alpha; IL-6: Interleukin-6; IL-8: Interleukin-8; IL-1β: Interleukin-1β; iNOS: Inducible Nitric Oxide Synthase; GADPH: Glyceraldehyde 3 Phosphate Dehydrogenase.

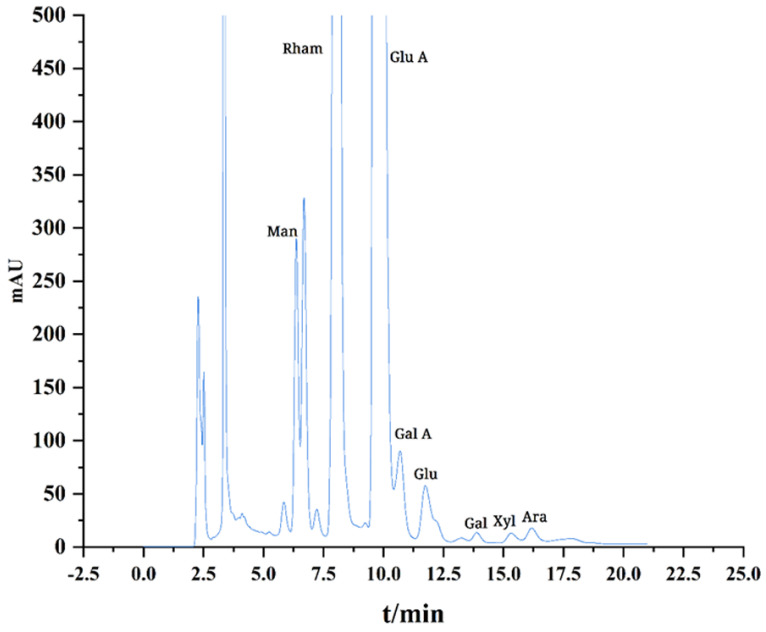

Figure 2. The glycuronic acid and monosaccharide components in IOP were studied by HPLC.

first extracts the total protein from uterine tissue cells and uses BCA for quantitative measurement. In addition, sodium lauryl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis can separate different molecular weights from proteins. First, an equal amount of protein was subjected to 10% SDS-PAGE gel electrophoresis, followed by transfer to a polyvinylidene fluoride membrane. At 25 degrees, the membrane in 5% milk-Tris buffer saline and BST at 0.1% Tween® 20 detergent were incubated together for 1 hour, and then incubated with primary antibodies and cultured at 4°C. Then, it was incubated with the secondary antibody (1:10000). Finally, the bands were visualized using the enhanced chemiluminescent kit. β-actin was used as the internal reference.

Statistical analysis

The statistical and analytical work of the data was done using the GraphPad Prism 8.2 software. The comparison of each set of data was based on the one-way analysis of variance with Tukey's multiple comparison post hoc test. The Kruskal-Wallis H test was used for non-normal variance, and Dunnett's T3 multiple comparisons test was used for homogeneity of variance. Data were expressed as mean ± SEM. $P < 0.05$ represented a statistically significant value.

Results

Monosaccharide and uronic acid composition of IOP

Figure 2 shows a chromatographic infographic of the IOP. It is clear that the purified polysaccharides are composed of Man, Rham, Ribose, Gal, Glu, Xyl, and Ara, respectively, with a molar ratio of 0.8:74:0.1:0.2:0.2:0.1:0.3. The molar ratio of Glu A:Gal A is 11:0.2.

The effect of IOP on mEEC viability

CCK-8 reagent detection method was used to investigate whether intraocular pressure had an effect on mEEC activity. From the results of **Figure 3**, IOP does not affect the activity of mEEC.

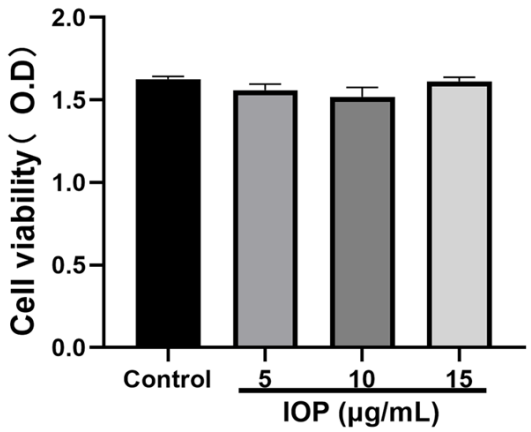

Figure 3. The effect of IOP on cell viability. Culture endometrial epithelial cells with 5, 10 and 15 μg/mL IOP solutions for 24 h. Cell viability was determined using the CCK-8 reagent assay.

The role of IOP in the histopathological changes of LPS-induced endometritis

Histopathological results revealed that the mice in the control and amoxicillin groups had normal uterine tissue morphology; mice in the LPS group exhibited uneven thickness of the endometrial epithelium, congestion, and swelling of several capillaries in the lamina propria and increased eosinophilia (**Figure 4**). However, treatment with IOP reduced these pathological effects, and the highest reduction was observed following high-dose IOP treatment.

The effect of IOP on MPO activity in LPS-induced endometritis

We determined MPO activity in the uterine tissue using ELISA. Compared with the control group, the activity of MPO was seen to be increased. Additionally, there was a considerable decrease in MPO activity following IOP treatment (**Figure 5**).

IOP impacted the inflammatory cytokines and mediator of LPS-induced endometritis

We derived protein expression components of TNF-α, IL-6, IL-1β, IL-8, and inducible nitric oxide synthase (iNOS) to better understand how IOP affects LPS-induced endometritis. The results revealed that, compared with the control group, there was an increase in the levels of TNF-α, IL-6, IL-1β, IL-8, and iNOS in the LPS group whereas a decrease in the expression levels of these factors in the IOP groups (**Figure 6**).

IOP affected the TLR4 signaling pathway in LPS-induced endometritis

TLR4 signal is an important factor in congenital inflammation. The binding of LPS to TLR4 can activate MyD88 and NF-κB, which causes the release of pro-inflammatory factors. **Figure 7A** and **7B** are the results of the analysis of western blotting, compared to the blank control group, there was an elevated expression of TLR4, MyD88, p-IκBα, and p-p65 in the LPS group, which can be reversed by IOP treatment.

Discussion

The histopathology of endometritis can be both acute and prolonged. Acute endometritis is characterized by the formation of superficial endometrium, microabscess in the glandular and uterine cavity, and neutrophil infiltration [36, 37]. Growing evidence indicates that IOP is involved in various inflammatory diseases and modulates adverse pregnancy reactions in mice induced by infection [38]. This study also tried to observe the pathological changes of the uterine tissue of mice, in order to better understand the role of intraocular pressure. Our results revealed that LPS induced the falling off of the endometrial epithelial cells and swelling of the endometrial epithelium and uterine gland epithelial cells with neutrophil infiltration in the uterus of the mice, and IOP treatment can significantly improve inflammation. Moreover, we observed a decline in MPO expression. MPO is a heme protein rich in neutrophils (PMN), which is considered a marker of PMN activity [39] in the uterus of mice that received IOP. It is concluded that the decrease in MPO activity indicates that the inflammatory symptoms are weakened, and it is verified that IOP can effectively improve the symptoms of endometritis induced by LPS.

LPS has been known to promote the release of inflammatory cytokines, such as TNF-α, IL-6, IL-1β, and IL-8 [40-42]. Cells releasing too many pro-inflammatory cytokines can exacerbate the body's immune response, which activates the inflammatory cascades and subsequently causes tissue injury [43-45]. Previous studies have reported that IOP can significantly reduce IL-6, TNF-α, and overall IL-1β protein expression. Our experimental results indicated that the expression of TNF-α, IL-6, IL-1β, and IL-8

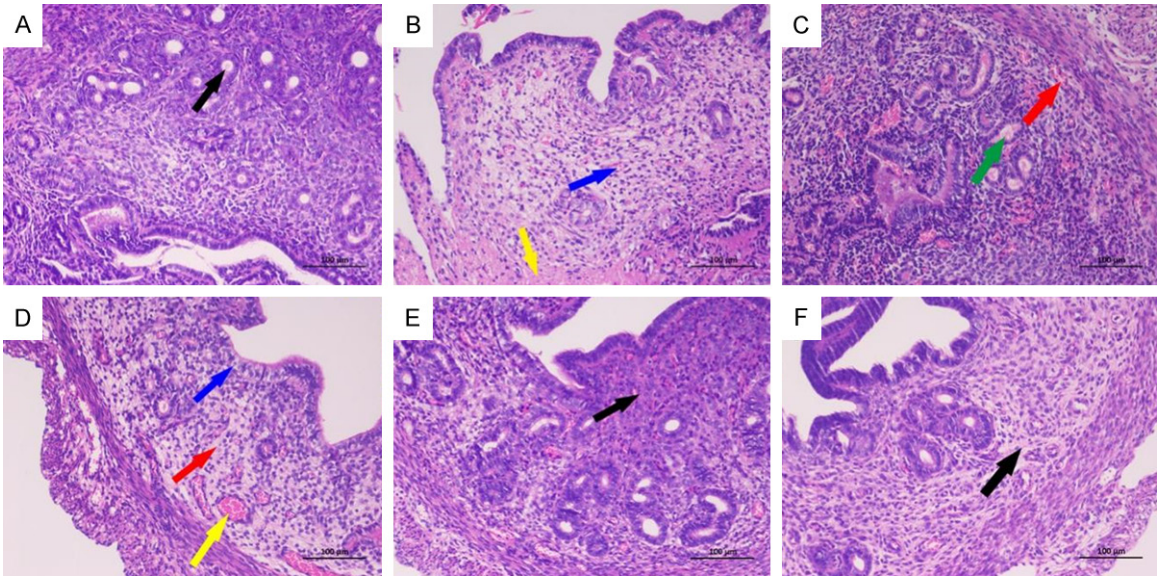

Figure 4. The impact of *Inonotus obliquus* polysaccharide (IOP) on lipopolysaccharide (LPS)-induced histopathological changes in the uterus. A. Control group. Eosinophilic secretions were observed in the uterine glands (black arrow). B. LPS group. Local lamina propria and muscular necrosis; the nuclei of the cells were fragmented and dissolved; eosinophils were enhanced (yellow arrow). A large amount of capillary blood extravasation was observed in the lamina propria (blue arrow). C. LPS+ low-dose IOP group. Numerous eosinophilic secretions (green arrows) and blood stasis (red arrows) were observed in the uterine glands. D. LPS+ medium-dose IOP group. A few endometrial epithelial cells were swollen, with loose cytoplasm (blue arrow), lamina propria edema, loose connective tissue arrangement (red arrow), and dilated blood vessels (yellow arrow). E. LPS+ high-dose IOP group. A few capillaries were congested (black arrow). F. LPS+ amoxicillin group. Lamina propria edema and loose connective tissue arrangement (black arrow).

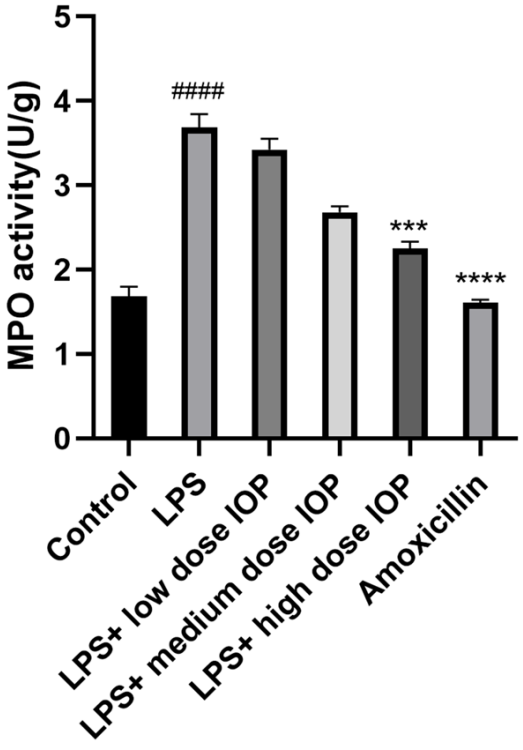

Figure 5. The effect of *Inonotus obliquus* polysaccharide (IOP) on lipopolysaccharide (LPS)-induced myeloperoxidase (MPO) activity. The values are represented as mean ± SEM (n=12 each) of three independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, and $^{\#\#\#\#}P < 0.0001$ vs control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ vs LPS group.

was increased after treatment with LPS; however, IOP treatment suppressed the expression of these factors. Additionally, inflammatory mediator iNOS plays an important role in LPS-induced inflammation [8, 46]. The expression of iNOS was increased following LPS stimulation albeit decreased after IOP treatment. These experimental results all point out that intraocular pressure has a good preventive treatment effect on the symptoms of endometritis induced by LPS.

Recently, the protective role of IOP in inflammation has been confirmed by inhibiting the TLR4 signaling pathway [38, 47]. TLR4 is a signal-transducing receptor used by gram-negative bacterial LPS and heat shock proteins. LPS cooperates with TLR4 by binding to LPS-binding protein (LBP) to form the CD14/MD2/TLR4 receptor complex [11]. TLR4 recognizes LPS, a

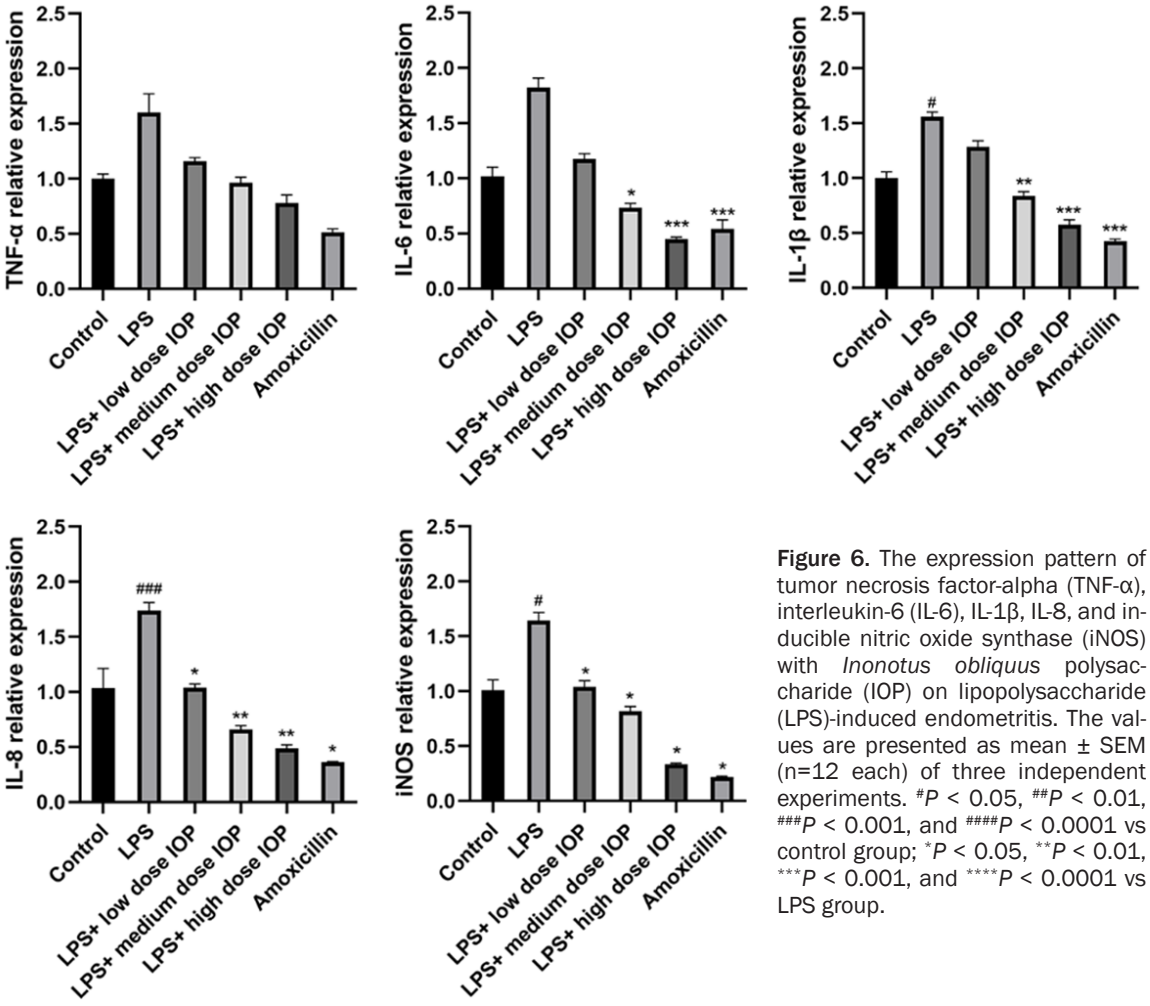

Figure 6. The expression pattern of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), IL-1β, IL-8, and inducible nitric oxide synthase (iNOS) with *Inonotus obliquus* polysaccharide (IOP) on lipopolysaccharide (LPS)-induced endometritis. The values are presented as mean ± SEM (n=12 each) of three independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, and $^{\#\#\#\#}P < 0.0001$ vs control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ vs LPS group.

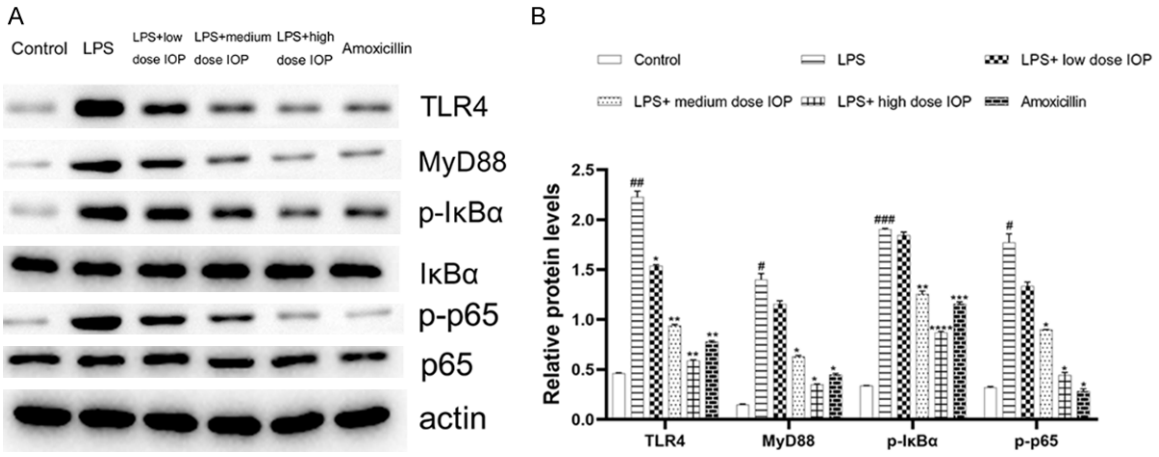

Figure 7. The effect of *Inonotus obliquus* polysaccharide (IOP) on toll-like receptor (TLR4) pathway activation. A. Western blotting. B. The expression of TLR4, myeloid differentiation factor 88 (MyD88), phosphorylated nuclear factor of kappa light polypeptide enhancer gene in B-cell inhibitor, alpha (p-IκBα), and p-p65 proteins in lipopolysaccharide (LPS)-induced uterine inflammation. β-actin was considered the internal control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, and $^{\#\#\#\#}P < 0.0001$ vs control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ vs LPS group.

major component of the gram-negative bacterial cell wall, through its N-terminal extracellular domain rich in leucine repeats. TLR4 binds to the junction protein via its C-terminal intracellular TIR domain and then mobilizes to undergo a signaling cascade to reduce the release of inflammatory factors [13]. MyD88 is a downstream pathway protein of TLR4. TLR4 causes the transcriptional activation of NF-κB via the MyD88 signaling pathways and plays an important role in inflammation treatment and immunoprevention [48, 49]. It is well-known that IκBα is an inhibitor of the NF-κB pathway and is phosphorylated and then degraded after the activation of NF-κB. Meanwhile, the activation of NF-κB promotes NF-κB p65 phosphorylation, causing the expression of downstream pro-inflammatory factors [50, 51]. Therefore, we hypothesized that IOP alleviates LPS-induced endometritis by inhibiting the TLR4/NF-κB pathway. Our results indicated that the expression of TLR4, MyD88, and p-IκBα changed. After LPS induction, the content of p-p65 increased. However, after IOP treatment, the p-p65 content was effectively reduced. From the change situation, IOP uses the control of TLR4/NF-κB signaling pathway to protect the symptoms of endometritis induced by LPS.

Owing to the strong biological characteristics of IOP, health products and tea derived from it have currently entered the market [52], however, the products are relatively simple. A thorough pharmacological analysis is required for its application as a potential drug. However, there is still room for improvement in the extraction and purification of IOP, and further work is needed to clarify its chemical formula, which is our next step. Additionally, we observed that IOP regulates the TLR4/NF-κB signaling pathway following LPS induction; however, the specific mechanism needs further exploration.

Conclusion

Conclusively, we demonstrated the protective function of IOP in LPS-induced endometritis. In this study, IOP improved LPS-induced pathological damage in endometritis and reduced MPO activity in tissues. Furthermore, IOP decreased the iNOS expression. It is worth emphasizing that intraocular pressure can regulate the signaling pathway of TLR4/NF-κB, prevent the release of pro-inflammatory cytokines, and have a strong anti-inflammatory effect. Therefore, the study of intraocular pressure is indeed of great help in the treatment and prevention of LPS-induced endometritis.

Acknowledgements

This work was supported by the Chengdu Normal University Scientific Research Project [No. 111-153701], the Public Welfare Project of Pet Chaperoning [No. 107-261277] and the Agricultural Ecology, and Green Food Development Project [CSCXTD2020B11]. The funders had no role in the study design, data collection and analysis, publication decision, or preparation of the manuscript.

Disclosure of conflict of interest

None.

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References

[1] Liu Y, Qiu C, Li W, Mu W, Li C and Guo M. Selenium plays a protective role in staphylococcus aureus-induced endometritis in the uterine tissue of rats. Biol Trace Elem Res 2016; 173: 345-353.

[2] Sheldon IM and Bromfield JJ. Innate immunity in the human endometrium and ovary. Am J Reprod Immunol 2011; 66 Suppl 1: 63-71.

[3] Turner ML, Healey GD and Sheldon IM. Immunity and inflammation in the uterus. Reprod Domest Anim 2012; 47 Suppl 4: 402-409.

[4] Czernobilsky B. Endometritis and infertility. Fertil Steril 1978; 30: 119-130.

[5] Kitaya K, Matsubayashi H, Takaya Y, Nishiyama R, Yamaguchi K, Takeuchi T and Ishikawa T. Live birth rate following oral antibiotic treatment for chronic endometritis in infertile women with repeated implantation failure. Am J Reprod Immunol 2017; 78.

[6] Andrews WW, Hauth JC, Cliver SP, Conner MG, Goldenberg RL and Goepfert AR. Association of asymptomatic bacterial vaginosis with endometrial microbial colonization and plasma cell endometritis in nonpregnant women. Am J Obstet Gynecol 2006; 195: 1611-1616.

[7] Kong F, Sun Y, Song W, Zhou Y and Zhu S. MiR-216a alleviates LPS-induced acute lung injury

via regulating JAK2/STAT3 and NF-kappaB signaling. Hum Cell 2020; 33: 67-78.
[8] Wu H, Zhao G, Jiang K, Li C, Qiu C and Deng G. Engeletin alleviates lipopolysaccharide-induced endometritis in mice by inhibiting TLR4-mediated NF-kappaB activation. J Agric Food Chem 2016; 64: 6171-6178.
[9] Nara K, Kawashima N, Noda S, Fujii M, Hashimoto K, Tazawa K and Okiji T. Anti-inflammatory roles of microRNA 21 in lipopolysaccharide-stimulated human dental pulp cells. J Cell Physiol 2019; 234: 21331-21341.
[10] Takeuchi O and Akira S. Pattern recognition receptors and inflammation. Cell 2010; 140: 805-820.
[11] Saferding V and Bluml S. Innate immunity as the trigger of systemic autoimmune diseases. J Autoimmun 2020; 110: 102382.
[12] Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B and Beutler B. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 1998; 282: 2085-2088.
[13] Kuzmich NN, Sivak KV, Chubarev VN, Porozov YB, Savateeva-Lyubimova TN and Peri F. TLR4 signaling pathway modulators as potential therapeutics in inflammation and sepsis. Vaccines (Basel) 2017; 5: 34.
[14] Akira S and Takeda K. Toll-like receptor signalling. Nat Rev Immunol 2004; 4: 499-511.
[15] Yan S, Wang P, Wang J, Yang J, Lu H, Jin C, Cheng M and Xu D. Long non-coding RNA HIX003209 promotes inflammation by sponging miR-6089 via TLR4/NF-kappaB signaling pathway in rheumatoid arthritis. Front Immunol 2019; 10: 2218.
[16] Ju M, Liu B, He H, Gu Z, Liu Y, Su Y, Zhu D, Cang J and Luo Z. MicroRNA-27a alleviates LPS-induced acute lung injury in mice via inhibiting inflammation and apoptosis through modulating TLR4/MyD88/NF-kappaB pathway. Cell Cycle 2018; 17: 2001-2018.
[17] Monga M and Oshiro BT. Puerperal infections. Semin Perinatol 1993; 17: 426-31.
[18] Zhao HX, Zhao JL, Shen JZ, Fan HL, Guan H, An XP and Li PF. Prevalence and molecular characterization of fluoroquinolone resistance in escherichia coli isolates from dairy cattle with endometritis in China. Microb Drug Resist 2014; 20: 162-169.
[19] Zhou M, Yi Y and Hong L. Oridonin ameliorates lipopolysaccharide-induced endometritis in mice via inhibition of the TLR-4/NF-kappaB pathway. Inflammation 2019; 42: 81-90.
[20] Wang X, Yuan T, Yin N, Ma X, Zhang Z, Zhu Z, Shaukat A and Deng G. Luteoloside protects the uterus from staphylococcus aureus-induced inflammation, apoptosis, and injury. Inflammation 2018; 41: 1702-1716.
[21] Li R, Maimai T, Yao H, Liu X, He Z, Xiao C, Wang Y and Xie G. Protective effects of polydatin on LPS-induced endometritis in mice. Microb Pathog 2019; 137: 103720.
[22] Ma L, Chen H, Dong P and Lu X. Anti-inflammatory and anticancer activities of extracts and compounds from the mushroom Inonotus obliquus. Food Chem 2013; 139: 503-508.
[23] Lu X, Chen H, Dong P, Fu L and Zhang X. Phytochemical characteristics and hypoglycaemic activity of fraction from mushroom Inonotus obliquus. J Sci Food Agric 2010; 90: 276-280.
[24] Choi SY, Hur SJ, An CS, Jeon YH, Jeoung YJ, Bak JP and Lim BO. Anti-inflammatory effects of Inonotus obliquus in colitis induced by dextran sodium sulfate. J Biomed Biotechnol 2010; 2010: 943516.
[25] Chen YF, Zheng JJ, Qu C, Xiao Y, Li FF, Jin QX, Li HH, Meng FP, Jin GH and Jin D. Inonotus obliquus polysaccharide ameliorates dextran sulphate sodium induced colitis involving modulation of Th1/Th2 and Th17/Treg balance. Artif Cells Nanomed Biotechnol 2019; 47: 757-766.
[26] Han Y, Nan S, Fan J, Chen Q and Zhang Y. Inonotus obliquus polysaccharides protect against Alzheimer's disease by regulating Nrf2 signaling and exerting antioxidative and anti-apoptotic effects. Int J Biol Macromol 2019; 131: 769-778.
[27] Tian J, Hu X, Liu D, Wu H and Qu L. Identification of Inonotus obliquus polysaccharide with broad-spectrum antiviral activity against multi-feline viruses. Int J Biol Macromol 2017; 95: 160-167.
[28] Hu Y, Sheng Y, Yu M, Li K, Ren G, Xu X and Qu J. Antioxidant activity of Inonotus obliquus polysaccharide and its amelioration for chronic pancreatitis in mice. Int J Biol Macromol 2016; 87: 348-356.
[29] Zhang L, Fan C, Liu S, Zang Z and Zhang L. Chemical composition and antitumor activity of polysaccharide from Inonotus obliquus. Journal of Medicinal Plants Research 2011; 5: 1251-1260.
[30] Lee JS, Lee KR, Lee S, Lee HJ, Yang HS, Yeo J, Park JM, Choi BH and Hong EK. Polysaccharides isolated from liquid culture broth of Inonotus obliquus inhibit the invasion of human non-small cell lung carcinoma cells. Biotechnol Bioprocess Eng 2017; 22: 45-51.
[31] Hu B, Dong Y, Zhou W, Ma Y, Li L, Fu X, Zhang W, Luo Y, Pu J, Deng X, Zhang R and Liu S. Effect of Inonotus obliquus polysaccharide on composition of the intestinal flora in mice with

acute endometritis. PLoS One 2021; 16: e0259570.
[32] Wu H, Dai A, Chen X, Yang X, Li X, Huang C, Jiang K and Deng G. Leonurine ameliorates the inflammatory responses in lipopolysaccharide-induced endometritis. Int Immunopharmacol 2018; 61: 156-161.
[33] Gargiulo S, Greco A, Gramanzini M, Esposito S, Affuso A, Brunetti A and Vesce G. Mice anesthesia, analgesia, and care, part I: anesthetic considerations in preclinical research. ILAR J 2012; 53: E55-E69.
[34] Yang M, Hu D, Cui Z, Li H, Man C and Jiang Y. Lipid-lowering effects of Inonotus obliquus polysaccharide in vivo and in vitro. Foods 2021; 10: 3085.
[35] Jiang K, Chen X, Zhao G, Wu H, Mi J, Qiu C, Peng X and Deng G. IFN-tau plays an anti-inflammatory role in staphylococcus aureus-induced endometritis in mice through the suppression of NF-kappaB pathway and MMP9 expression. J Interferon Cytokine Res 2017; 37: 81-89.
[36] Kitaya K, Takeuchi T, Mizuta S, Matsubayashi H and Ishikawa T. Endometritis: new time, new concepts. Fertil Steril 2018; 110: 344-350.
[37] Haggerty CL, Ness RB, Amortegui A, Hendrix SL, Hillier SL, Holley RL, Peipert J, Randall H, Sondheimer SJ, Soper DE, Sweet RL and Trucco G. Endometritis does not predict reproductive morbidity after pelvic inflammatory disease. Am J Obstet Gynecol 2003; 188: 141-148.
[38] Xu L, Yu Y, Sang R, Ge B, Wang M, Zhou H and Zhang X. Inonotus obliquus polysaccharide protects against adverse pregnancy caused by Toxoplasma gondii infection through regulating Th17/Treg balance via TLR4/NF-κB pathway. Int J Biol Macromol 2020; 146: 832-840.
[39] Borregaard N, Sørensen OE and Theilgaard-Monch K. Neutrophil granules: a library of innate immunity proteins. Trends Immunol 2007; 28: 340-345.
[40] Wang X and Zhang Y. Resveratrol alleviates LPS-induced injury in human keratinocyte cell line HaCaT by up-regulation of miR-17. Biochem Biophys Res Commun 2018; 501: 106-112.
[41] Baggiolini M and Clark-Lewis I. Interleukin-8, a chemotactic and inflammatory cytokine. FEBS Lett 1992; 307: 97-101.
[42] Li S, Xie R, Jiang C and Liu M. Schizandrin A alleviates LPS-induced injury in human keratinocyte cell hacat through a microRNA-127-dependent regulation. Cell Physiol Biochem 2018; 49: 2229-2239.
[43] Zhao G, Jiang K, Wu H, Qiu C, Deng G and Peng X. Polydatin reduces Staphylococcus aureus lipoteichoic acid-induced injury by attenuating reactive oxygen species generation and TLR2-NFkappaB signalling. J Cell Mol Med 2017; 21: 2796-2808.
[44] Knapp S, Florquin S, Golenbock DT and van der Poll T. Pulmonary lipopolysaccharide (LPS)-binding protein inhibits the LPS-induced lung inflammation in vivo. J Immunol 2006; 176: 3189-3195.
[45] Chen H, Bai C and Wang X. The value of the lipopolysaccharide-induced acute lung injury model in respiratory medicine. Expert Rev Respir Med 2010; 4: 773-783.
[46] Chen YF, Wang YW, Huang WS, Lee MM, Wood WG, Leung YM and Tsai HY. Trans-cinnamaldehyde, an essential oil in cinnamon powder, ameliorates cerebral ischemia-induced brain injury via inhibition of neuroinflammation through attenuation of iNOS, COX-2 expression and NFκ-B signaling pathway. Neuromolecular Med 2016; 18: 322-333.
[47] Sang R, Sun F, Zhou H, Wang M, Li H, Li C, Sun X, Zhao X and Zhang X. Immunomodulatory effects of Inonotus obliquus polysaccharide on splenic lymphocytes infected with Toxoplasma gondii via NF-κB and MAPKs pathways. Immunopharmacol Immunotoxicol 2022; 44: 129-138.
[48] Pang Z, Junkins RD, Raudonis R, MacNeil AJ, McCormick C, Cheng Z and Lin TJ. Regulator of calcineurin 1 differentially regulates TLR-dependent MyD88 and TRIF signaling pathways. PLoS One 2018; 13: e0197491.
[49] Kim AY, Shim HJ, Kim SY, Heo S and Youn HS. Differential regulation of MyD88- and TRIF-dependent signaling pathways of toll-like receptors by cardamonin. Int Immunopharmacol 2018; 64: 1-9.
[50] Fitzgerald KA and Chen ZJ. Sorting out toll signals. Cell 2006; 125: 834-836.
[51] Gluba A, Banach M, Hannam S, Mikhailidis DP, Sakowicz A and Rysz J. The role of Toll-like receptors in renal diseases. Nat Rev Nephrol 2010; 6: 224-235.
[52] Lu Y, Jia Y, Xue Z, Li N, Liu J and Chen H. Recent developments in Inonotus obliquus (chaga mushroom) polysaccharides: isolation, structural characteristics, biological activities and application. Polymers (Basel) 2021; 13: 1441.