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RESEARCH ARTICLE

Morin Inhibits Inflammation, Oxidative Stress and Ferroptosis in Lipopolysaccharide-Induced Endometritis

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ABSTRACT

Endometritis is a uterine condition that results in significant detriment to the advancement of the global dairy industry. Morin is a phyto-flavonoid compound known for its diverse biological functions. The effect of morin on LPS-triggered endometritis remained undetermined. In the current study, sixty adult mice were allocated to four (*n*=15) treatment groups, i.e., Control, LPS, LPS + Morin, and Morin groups. Intrauterine infusion of LPS was used to establish an endometritis model in mice. An intraperitoneal injection of morin was administered to the mice 1 hour before an intrauterine infusion of LPS. After 24h of intrauterine injection of LPS, all the mice were euthanized. Our results revealed that morin rescued uterine injury by relieving the histopathological alterations caused by LPS. Morin significantly ($P<0.05$) reduced myeloperoxidase and pro-inflammatory cytokines following LPS exposure. It significantly ($P<0.05$) attenuated malondialdehyde production and iron accumulation while markedly enhanced glutathione and adenosine triphosphate levels in response to LPS exposure. Furthermore, morin treatment significantly increased mRNA and protein expression of TLR4. Moreover, it significantly ($P<0.05$) repressed TLR4 and down streamed NF-κB signaling. Notably, morin ($P<0.05$) suppressed LPS-induced oxidative damage by scavenging ROS and enhancing antioxidant enzymes activity. Additionally, morin activated the Nrf2 pathway, which was downregulated by LPS, which enhanced the antioxidant defence system. Our findings demonstrated that morin confers protection against endometritis in mice by inhibiting inflammation, oxidative stress, and ferroptosis.

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INTRODUCTION

Endometritis is a common reproductive disorder caused by several factors, including bacterial contamination in the dairy industry globally. Endometritis is linked to a decrease in reproductive performance due to low conception rates, premature embryo death, and extended calving intervals, resulting in considerable economic losses owing to low milk yield and various other health risks (Kasimanickam *et al.,* 2013). Endometritis is induced mainly by the invasion of infectious bacteria into the uterine cavity and most likely arises after parturition, unhygienic artificial insemination, vaginitis, and cervicitis. Reddish brown, foul-smelling uterine secretions, fever, dehydration, and depression are the characteristic features of endometritis (Wu *et al.,* 2016). *Escherichia* (*E.*) coli has been identified as the predominant pathogenic bacterium associated with uterine diseases (Piras *et al.,* 2017). Lipopolysaccharide (LPS) is derived from the cell wall of Gram-negative (G-ve) bacteria. The uterine endometrium identifies pathogen-associated molecular patterns (PAMPs) to initiate the immunological process (Jiang *et*

al., 2019). Exogenous PAMPs like LPS trigger the activation of Toll-like receptor-4 (TLR4) (Han *et al.,* 2016). The NF-κB pathway is stimulated due to TLR4 activation in response to LPS (Wu *et al.,* 2016). Inflammatory cytokine production is being induced following stimulation of the TLR4-mediated NF-κB pathway (Liu *et al.,* 2020).

Chronic inflammation elicits reactive oxygen species (ROS) production and induces oxidative stress. ROS initiates the activation of antioxidant enzymes, including heme oxygenase (HO)-1, which is regulated by nuclear factor erythroid 2-related factor 2 (Nrf2) (Lv *et al.,* 2016). Several researchers have explored the significant role of ROS in Nrf2 and NF-κB signaling (Jiang *et al.,* 2021; Shaukat *et al.,* 2024). Ferroptosis is a nonapoptotic-regulated cell death mechanism based on iron and lipids (Jiang *et al.,* 2021). Studies have shown that ferroptosis plays a significant role in the pathogenesis of inflammatory disorders (Bao *et al.,* 2022; Shen *et al.,* 2023), such as endometritis (Wang *et al.,* 2022; Cao *et al.,* 2023). Similarly, LPS also modulates Nrf2 pathway genes (Jiang *et al.,* 2021; Shauk*at et al.,* 2022).

Currently, uterine diseases, including endometritis of bacterial origin, have been treated with antibiotics. Excessive misuse of routinely used antibiotics might result in antibiotic resistance and serious food safety issues due to antibiotic residues in milk and meat (Shaukat *et al.,* 2021). Therefore, the present era desperately needs to discover novel preventive and therapeutic alternative natural compounds to treat endometritis. Several previous studies have utilized the mouse model (Wu *et al.,* 2016; Jiang *et al.,* 2021) because of several benefits over dairy animals, including ease of management and handling, low doses of medicine required, and a rapid reproduction rate.

Flavonoids are a large group of natural products that comprise flavones, isoflavones, flavonols, and flavanones. Morin (Fig. 1A) is a flavonol flavonoid, a yellowish compound naturally obtained from members of the *Moraceae* plant family. Morin is also obtained from red wine, onion, sweet chestnut, guava, almond, etc. (Caselli *et al.,* 2016; Lee *et al.,* 2016). Morin is a biologically active substance with antioxidant or free radical scavenging properties. Additionally, it exhibits antioxidant, antidiabetic, anti-inflammatory, antitumoral, antihypertensive, antimicrobial, hypouricemic, and neuroprotective properties by modulating the activity of various molecules (Caselli *et al.,* 2016; Rajput *et al.,* 2021). Nevertheless, morin's effect against LPS-triggered endometritis has not yet been investigated. Here, the LPS-induced murine endometritis model was used to explore the protective effect of morin. This study represents the first investigation into morin's preventive role against LPS-induced endometritis.

MATERIALS AND METHODS

Reagents: Morin (#B21110) was obtained from Yuan-ye Biotech. (Shanghai, China). LPS (#L2880) was supplied by Sigma (MO, USA). The ELISA kits for the detection of ROS (#E004-1-1), MPO (#A044-1-1), MDA (#A—3-1-2), SOD (#A001-3-2), CAT (#A007-1-1), Gpx1 (#A005-1-2), NO (#A012-1-2), ATP (#A095-1-1), GSH (#A006-2-1), and Fe^{2+} (#A039-2-1) were bought from Nanjing Jiangcheng (Nanjing, China). TNF-α (#430907), IL-6 (#575709), and IL-1β (#432601) were acquired from BioLegend (California, USA). TLR4 (#14378), NF-κB p65 (#8242), phospho-NF-κB p65 (#13346), IκB-α (#9242), phospho-IκB-α (#2859), Nrf2 (#12721), HO-1 (#86806), NQO1 (#3187), and β-actin (#3700) were procured from CST (Danvers, MA, USA) and GCLC (#ab190685) were bought from Abcom (Cambridge, MA, USA). All other reagents used in this study conformed to commercially available high-quality standards.

Compliance with ethical standards and animal welfare: *Ad libitum* feed and drinking water were provided to all groups of mice. The institutional research ethics and welfare committee approved this study.

Chromatography investigation of morin: Chromatography was carried out to calculate the purity of morin. Echrom2000 DAD data system was used in HPLC. The Hyper ODS2 C18 column was used in chromatography. The acetonitrile: water (2:98) was used in the elution process at a flow rate of 1ml/minute (min) at 295 nm. The purity of morin was 98% (Fig. 1B).

Mice and establishment of the endometritis model: Sixty female mice of the Kunming strain (weight range: 23-27grams) were randomly divided into four (n=15) groups. To elucidate the mechanism of endometritis induced by LPS, 50μL of LPS at 1mg/mL concentration was infused in intrauterine (JWu *et al.,* 2016; iang *et al.,* 2021). The mice were administered morin (50mg/kg) once 1hr before LPS treatment via the intraperitoneal route. After 24h, all the experimental mice were euthanized; uterine samples were collected and preserved at -80°C for subsequent tests. The diagrammatic overview of mouse treatment is presented in Fig. 1C.

Wet/dry ratios of uterine sample: The wet (W) weights of the uterus were initially recorded immediately after washing three times with PBS. The dry (D) weight of the uterus was recorded after incubating the uterus for 24h in an oven at 80°C (Liu *et al.,* 2020; Shaukat *et al.,* 2022). The W/D ratio determines the inflammatory edema of the uterus.

Histopathological examination: The histopathological assay measured the LPS-induced pathological lesions in uterine tissue. H&E staining was performed following a process that has been previously described (Gao *et al.,* 2024). Briefly, uterus tissue sections were immersed for 24h in a 4% paraformaldehyde solution. After this, the sections were subjected to dehydration, and liquid paraffin was used for embedding. The uterine samples were cut into 5μm pieces and then stained with H&E stains for microscopic examination.

Determination of inflammatory, oxidative stress, and ferroptosis markers: The homogenized uterine tissues were prechilled and centrifuged. The inflammatory (IL-1β, MPO, IL-6, NO, and TNF-α), oxidative stress (ROS, CAT, Gpx, and SOD), and ferroptosis (MDA, Fe^{2+}, ATP, and GSH) markers were determined in the supernatant using respective ELISA kits according to manufacturer instruction.

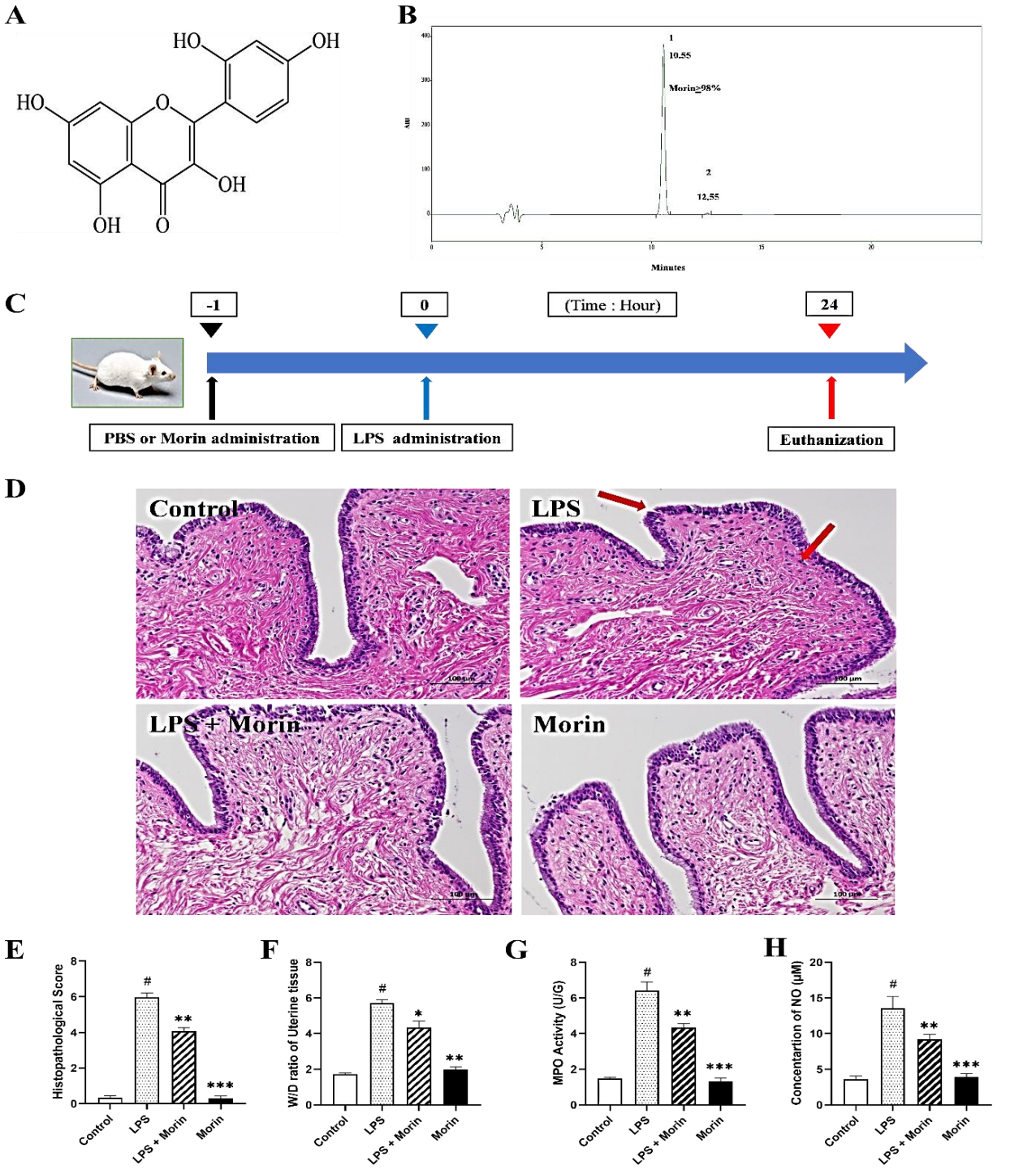

Fig. 1: Effect of morin on uterine injury caused by LPS. (A) Morin: chemical structural formula. (B) HPLC chromatogram. (C) Experimental design. (D) Histopathological sections of various experimental groups. Red arrows indicate inflammatory alterations. (E) Histological scoring. (F) W/D ratio. (G) MPO assay. (H) Nitric oxide concentration. PBS, phosphate-buffered saline; LPS, lipopolysaccharide; # P<0.001 vs. control group; * P<0.05, ** P<0.01, *** P<0.001 vs. the LPS group.

RT-qPCR: TRIzol was utilized to isolate all the RNAs from the sample. RNA samples were evaluated for concentration and purity using the NanoDrop 2000, which relies on the absorbance ratio at 260 and 280nm. Subsequently, cDNA was synthesized. Table 1 shows a list of primers. The mRNA expression of genes was determined using GAPDH as a reference gene. As previously described, the results were investigated by the $2^{-\Delta\Delta CT}$ method (Rajput *et al.*, 2019; Wang *et al.*, 2023).

Immunohistochemical (IHC) staining: The methods used for fixation, embedding, and slicing the uterine sections were the same as those used for H&E staining. Xylene was employed to deparaffinize tissue sections, which were thereafter treated for 10min at room temperature with 3% H_2O_2. Goat serum was used to block the area for 30min at 37°C. An overnight primary antibody incubation at 4°C was provided. Thereafter, secondary antibodies were applied for 2h at room temperature. After DAB staining, the uterine tissue sections were examined under a microscope.

Table 1: Primer sequence used in this study

Genes	Sequence (Forward to reverse)	Accession No.	Product Size
TLR4	ATTCAGAGCCGTTGGTGTATC GGGACTTCTCAACCTTCTCAAG	NM_021297.2	109
TNF-α	GGGCTTTACCTCATCTACTCA GCTCTTGATGGCAGACAGG	NM_013693.3	198
IL-1ß	CCTGGGCTGTCCTGATGAGAG TCCACGGGAAAGACACAGGTA	NM_008361.4	131
IL-6	GGCGGATCGGATGTTGTGAT GGACCCCAGACAATCGGTTG	NM_031168.1	199
SOD1	GGTCTCCAACATGCCTCTCT AACCATCCACTTCGAGCAGA	NM_011434.2	203
CAT	CACTGACGAGATGGCACACT TGTGGAGAATCGAACGGCAA	NM_009804.2	175
Gpx1	GTACTTGGGGTCGGTCATGA GGTTTCCCGTGCAATCAGTT	NM_001329527.1	222
Nrf2	TCCTATGCGTGAATCCCAAT GCGGCTTGAATGTTTGTCTT	NM_010902.3	103
HO-1	GGGCTGTGAACTCTGTCCAATGT TTGGTGAGGGAACTGTGTCAGG	NM_010442.2	162
NQO1	TTCTGTGGCTTCCAGGTCTTAG GTCAAACAGGCTGCTTGGAGCAA	NM_008706.5	156
GCLC	ACAAGGACGTGCTCAAGTGG CCAGGCGTTCCTTCGATCAT	NM_010295.2	199
GAPDH	CAATGTGTCCGTCGTGGATCT GTCCTCAGTGTAGCCCAAGATG	NM_001289726.1	124

Immunoblotting analysis: Western blotting was conducted in accordance with previously described procedures (Gao *et al.*, 2024). Briefly, total protein was isolated from uterine tissue by RIPA lysis buffer. Subsequently, the proteins were isolated via SDS-PAGE and fractionated to uniform protein concentrations. After that, proteins were transferred to a PVDF membrane, and the nonprotein sections were blocked by incubation with 5% skim milk solution for 2h. Subsequently, an overnight priming with primary antibodies was performed at 4°C. Thereafter, secondary antibodies were applied for 1h at room temperature. Western blot detection was carried out using an ECL Plus system to determine protein expression levels.

Statistical data analysis: The data was analysed by GraphPad Prism 9.4.0, and presented as the means±S.E.M.s. Statistical analysis was conducted using one-way ANOVA, followed by Dunnett's multiple comparison test. P values less than 0.05 were deemed indicative of statistical significance.

RESULTS

Impact of morin treatment against LPS-induced murine endometritis: The control group did not exhibit any inflammatory changes in the uterine parenchyma. The LPS caused significant uterine injury, as evidenced by hyperemia, hemorrhage, and a massive influx of inflammatory cells. However, morin significantly reversed the pathogenic changes induced by LPS (Fig. 1D). Histopathological sections were scored to assess histological alterations (Fig. 1E). To quantify the severity of inflammatory endometrial edema, we measured the W/D ratio, which was increased significantly (P<0.05) in response to LPS exposure. The morin treatment significantly reduced the W/D ratio (Fig. 1F). MPO value was greatly boosted in the LPS group, whereas morin administration decreased MPO activity (Fig. 1G). Nitric oxide (NO) generation was markedly increased under LPS exposure. However, morin distinctly downregulated LPS-triggered NO production, as illustrated in Fig. 1H.

Impact of morin on the synthesis of pro-inflammatory mediators: The effect of morin against LPS-triggered pro-inflammatory cytokines was examined using ELISA and qRT-PCR. LPS significantly boosted IL-1β, IL-6, and TNF-α as compared to the control group. The data showed that morin treatment (P<0.05) reduced the upregulation of pro-inflammatory mediators (Fig. 2A, B).

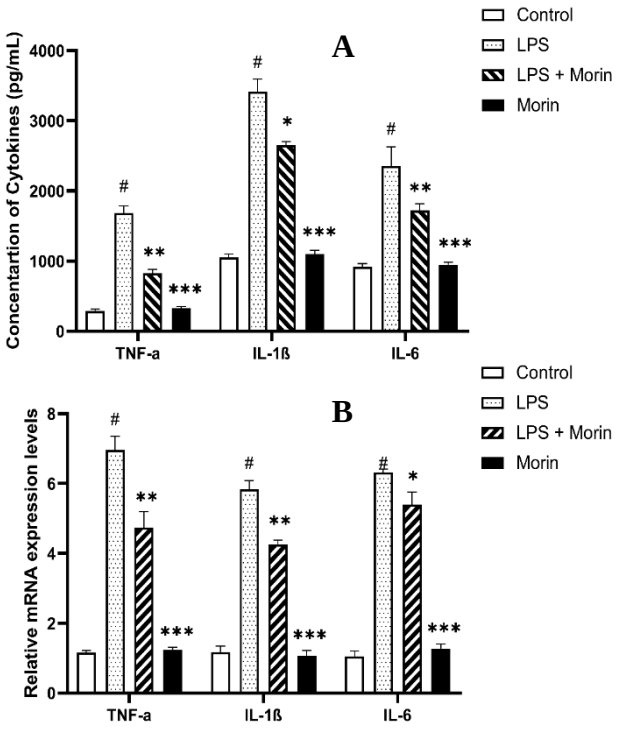

Fig. 2: Effect of morin on inflammatory response. (A) Concentrations of pro-inflammatory cytokines. (B) Relative mRNA expression of pro-inflammatory cytokines. # P<0.001 vs. the control group and * P<0.05, ** P<0.01, *** P<0.001 vs. the LPS group.

Morin inhibited LPS-induced ferroptosis: MDA, iron contents, ATP, and GSH were determined to assess the impact of morin on ferroptosis. The LPS group markedly (P<0.05) decreased the ATP and GSH levels and increased the levels of MDA and iron. However, the morin group showed reduction in MDA and iron contents and increased ATP and GSH levels (Fig. 3A-D). These results depicted that morin could prevent LPS initiated ferroptosis in the murine uterus.

Morin attenuated activation of NF-κB pathway: The expression of TLR4 in the LPS group was markedly increased (P<0.05) (Fig. 4A, B). Moreover, morin administration significantly reduced LPS-induced TLR4 expression (P<0.05). The impact of morin on LPS-induced endometritis was examined using western blotting to detect NF-κB pathway protein expression. The results indicated that the LPS group had considerably greater protein expression of TLR4, phosphorylated NF-κB p65, and IκB-α than the control group (Fig. 4C, D). However, morin therapy effectively reversed these alterations induced by LPS (P<0.05).

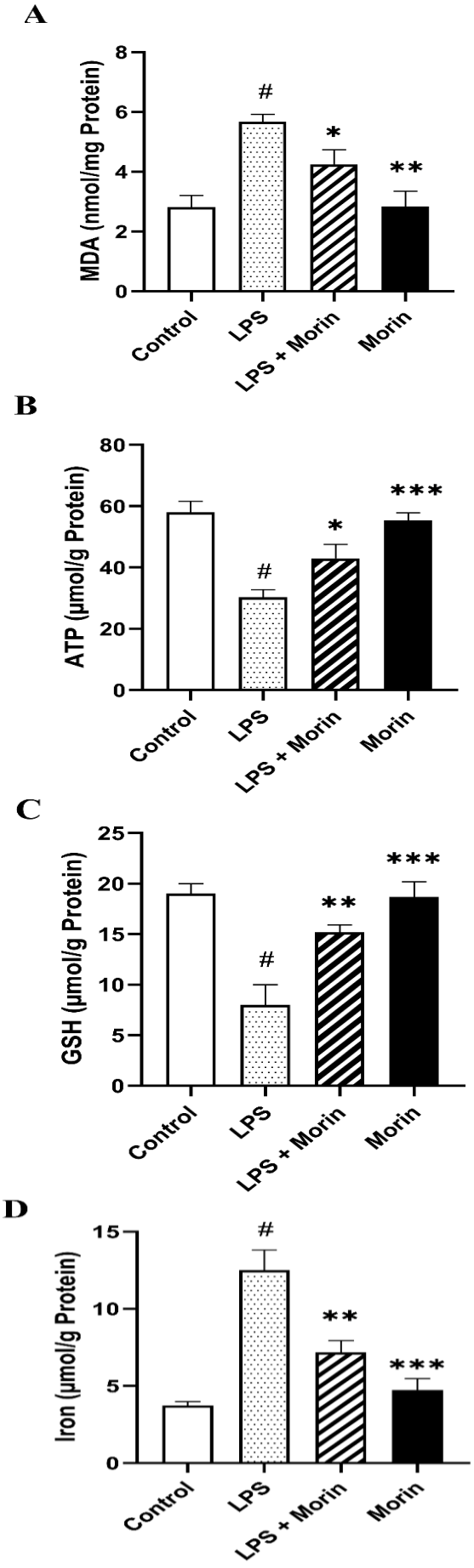

Fig. 3: Morin alleviates LPS-triggered ferroptosis. (A) MDA. (B) ATP. (C) GSH. (D) Iron concentration. # P<0.001 vs. the control group and * P<0.05, ** P<0.01, *** P<0.001 vs. the LPS group.

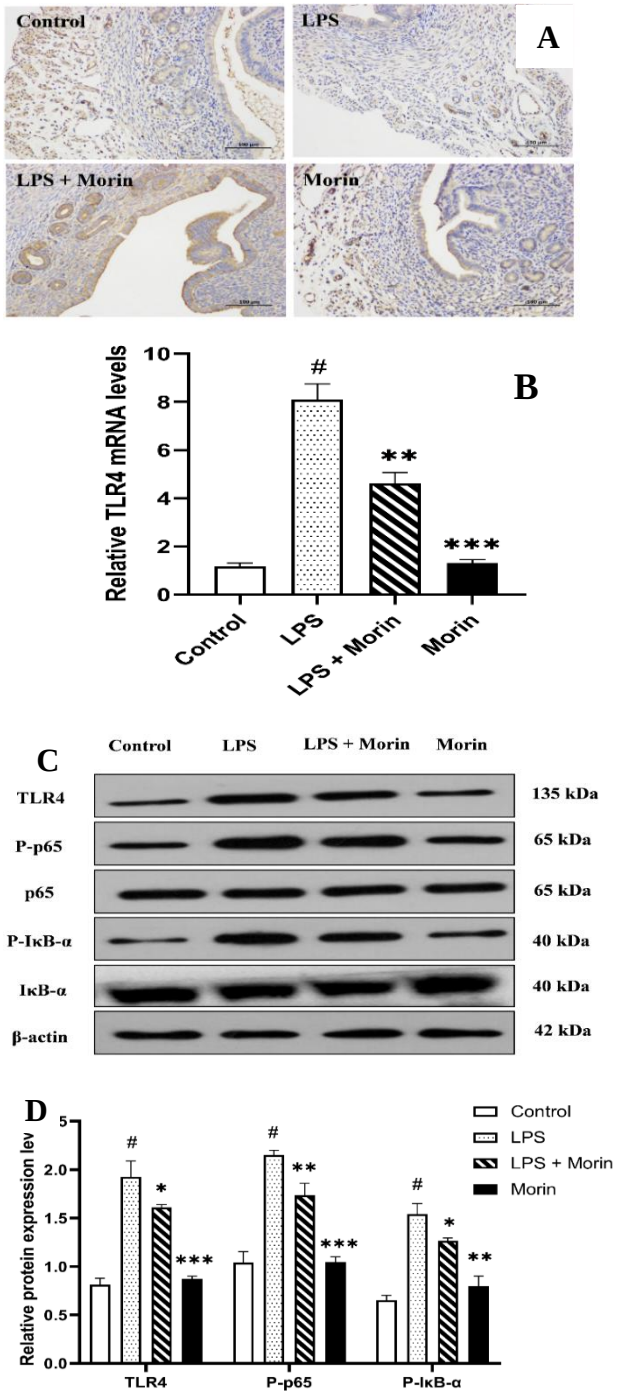

Fig. 4: Morin inhibited TLR4 and downstream NF-κB signaling activation. (A) TLR4 immunohistochemistry. (B) Gene expression of TLR4. (C) Representative WB bands showing TLR4 and the NF-κB pathway proteins. (D) Quantification graph. # P<0.001 vs. the control group and * P<0.05, ** P<0.01, *** P<0.001 vs. the LPS group.

Morin ameliorates oxidative damage triggered by LPS: LPS-exposed mice exhibited a substantial increase in ROS production (Fig. 5A). Concurrently, SOD, CAT, and Gpx1 activities were suppressed in the LPS group. However, morin administration dramatically reduced ROS accumulation and enhanced activities of CAT, SOD, and Gpx1 than the LPS group. RT-qPCR analysis revealed a decrease (P<0.05) in the relative mRNA abundance of antioxidant genes (SOD1, CAT, and GPx1) in the LPS group. Whereas it was observed that morin therapy significantly upregulated the mRNA expression of antioxidant enzymes (Fig. 5B).

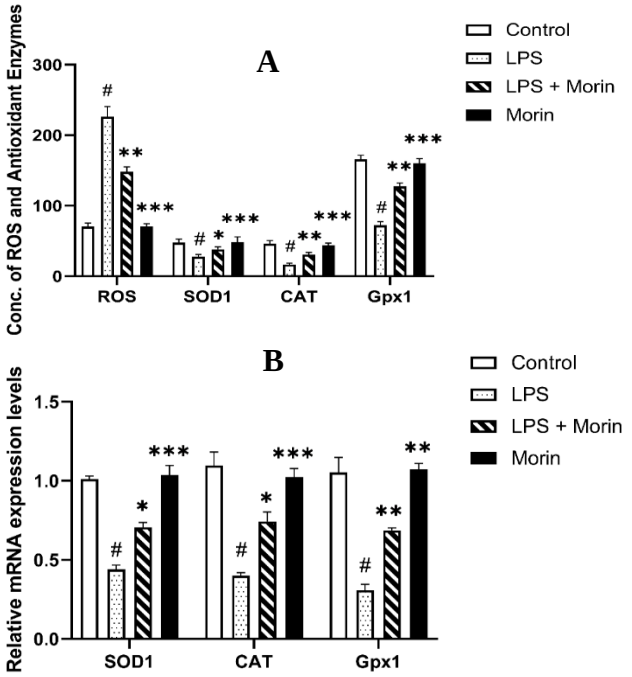

Fig. 5: Effect of morin LPS-triggered expression of ROS and antioxidant enzymes (A) Concentration of ROS and antioxidant enzymes (B) mRNA expression of antioxidant enzymes. # P<0.001 vs. the control group and * P<0.05, ** P<0.01, *** P<0.001 vs. the LPS group.

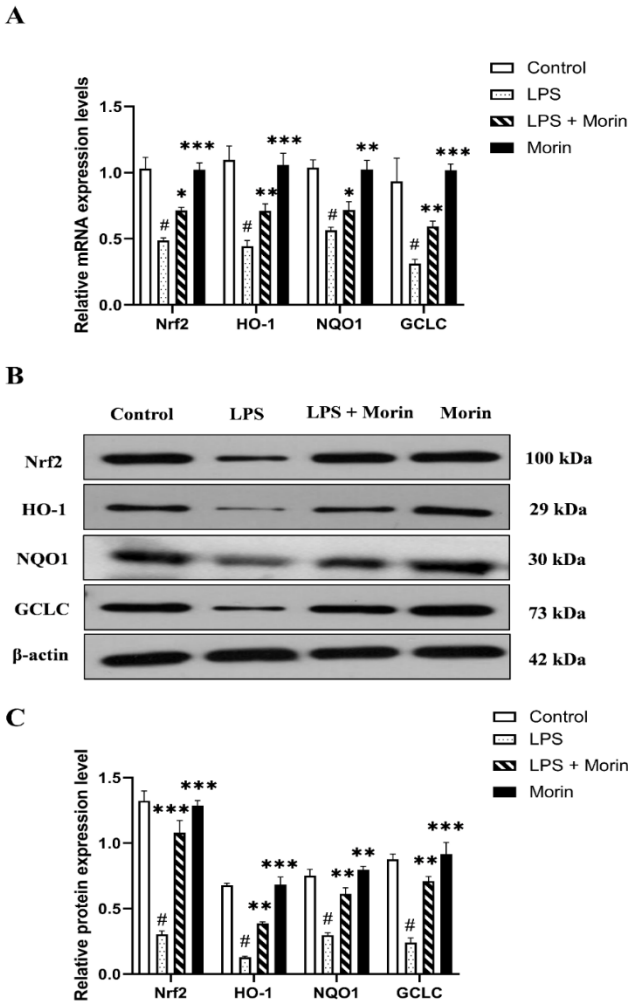

Fig. 6: Effect of morin in LPS-induced Nrf2 pathway. (A) mRNA expression of Nrf2 genes. (B) Representative WB bands of Nrf2 pathway genes. (C) Quantification. The control was β-actin. # P<0.001 vs. the control group and * P<0.05, ** P<0.01, *** P<0.001 vs. the LPS group.

Morin protects uterine tissue via upregulation of the Nrf2 pathway: The LPS-exposed group showed significantly lower (P<0.05) levels of Nrf2, HO-1, GCLC, and NQO1 mRNA and protein expression than the control group. However, it was dramatically (P<0.05) reversed by morin therapy (Fig. 6A, 6B, 6C). Our results showed that morin could reduce oxidative damage caused by LPS by stimulating the Nrf2 pathway.

DISCUSSION

Inflammation is recognized as an essential factor in the etiology of endometritis. Currently, antibiotics are used as primary therapeutic agents to treat endometritis; however, they are effective but cause bacterial resistance and food safety issues (Ibraimi *et al.,* 2015). Therefore, exploring safe and effective treatments for endometritis is crucial. Traditional Chinese remedies have been utilized worldwide to prevent and treat various illnesses. Morin is the major bioactive compound found in plants of the *Rosaceae, Moraceae,* and *Fagaceae* families. It is a polyphenolic molecule with vast biological potential, including regulating redox status via the Nrf2 pathway and preventing the generation of pro-inflammatory mediators through NF-κB regulation (Rajput *et al.,* 2021). LPS triggers inflammatory pathogenic reactions (Lv *et al.,* 2015; Zhang *et al.,* 2019; Yin *et al.,* 2019). However, no previous study has explored the impact of morin on endometritis triggered by LPS.

The pathological alterations in the uterus, such as inflammatory cell accumulation and sluffing of the epithelial lining of the endometrium, were observed in mice with LPS-induced endometritis. Moreover, preventive morin therapy one hour before LPS treatment significantly reduced these pathogenic alterations. These results indicate that morin stops endometritis caused by LPS. An obvious number of neutrophils and macrophages reach this location and intensify inflammation. Our findings demonstrated that morin significantly reduced MPO, NO, and W/D in endometritis caused by LPS. These findings are consistent with previous studies (Tianzhu *et al.,* 2014; Liu *et al.,* 2020; Shaukat *et al.,* 2022).

IL-1β and TNF-α are the key pro-inflammatory cytokines produced by immune cells (Liu *et al.,* 2024), active monocytes, and epithelial cells (Jiang *et al.,* 2018), and have been shown to activate the synthesis of additional inflammatory mediators. Pro-inflammatory cytokines activate cell adhesion molecules, which increase leukocyte migration and adherence to sites of inflammation. Surprisingly, morin therapy significantly suppressed the synthesis of pro-inflammatory cytokines. Our findings are in line with those of previous research studies (Lee *et al.,* 2008; Abd El-Aal *et al.,*2022). Subsequently, it was demonstrated that morin exerts its beneficial impact in endometritis by mitigating the synthesis of pro-inflammatory cytokines.

LPS is widely considered as to provoke immune responses primarily via activation of TLR4 and associated downstream NF-κB pathway (Lv *et al.,* 2015; Li *et al.,* 2015; Zhang *et al.,* 2019; Yin *et al.,* 2019). Previously, it has been confirmed that LPS enhances cytokine synthesis

by activating the TLR4 signaling pathway (Wu *et al.*, 2016). Our findings revealed that LPS significantly upregulated TLR4 expression, which was attenuated by morin treatment, suggesting the beneficial effects of morin through suppressing TLR4-mediated NF-κB activation.

Ferroptosis has been implicated in the pathogenesis of several inflammatory disorders, such as colitis, mastitis, and acute lung injury (Dixon *et al.,* 2012; Guo *et al.*, 2022; Zhu *et al.,* 2022; Zhang *et al*., 2023). This study determined the potential of morin to diminish endometritis severity through inhibition of ferroptosis in uterine tissue, as consistent with previous findings (Wang *et al.*, 2022; Jiang *et al.,* 2023; Zhao *et al.*, 2023; Gao *et al.*, 2024). Our findings demonstrated that morin could reduce LPS-induced ferroptosis in murine endometritis by restoring GSH and ATP levels while reducing iron and MDA accumulation.

The LPS administration significantly increased ROS production in the mouse uterus, while morin treatment effectively ameliorated this effect, consistent with previous studies (Bachewal *et al.,* 2018; Ulla *et al.*, 2021). Several antioxidant enzymes, such as SOD, CAT, and Gpx, rescue cells from oxidative damage (Chen *et al.,* 2011). A previous study demonstrated that morin could alleviate oxidative stress by enhancing SOD, CAT and GPx activities (Çelik *et al.,* 2020). Morin administration significantly upregulated the expression of SOD, CAT, and GPx in the murine uterus. These findings suggest that morin suppresses LPS-induced ROS-mediated oxidative stress by enhancing uterine antioxidant defence. The activation of the Nrf2 signaling pathway augments the transcription of antioxidant genes, including HO-1, NQO1, and GCLC, thus boosting cellular defense against oxidative stress (Jiang *et al.*, 2018; Shaukat *et al.*, 2022). Previous research indicates that morin prevents neuroinflammation, synovitis, and oxidative stress through activation of the Nrf2 signaling pathway (Arab *et al.*, 2020; Hussain *et al.*, 2020). The accurate mechanism underlying morin's attenuation of LPS-induced oxidative damage remains unclear; however, based on the results of this study, it could be declared that morin markedly enhanced LPS-induced expression of Nrf2, GCLC, HO-1 and NQO1 supporting its role in mitigating oxidative stress via activation of the Nrf2-mediated antioxidant pathway (Arab *et al.*, 2020; Hussain *et al.*, 2020).

Conclusions: In conclusion, LPS may exacerbate inflammation, oxidative stress, and ferroptosis in the murine uterus. Prophylactic administration of morin inhibited LPS-induced endometritis by attenuating pathological damage to the uterus, inflammation, oxidative stress, and ferroptosis by modulating the TLR4-associated NF-κB and Nrf2 signaling. Overall, this study provides insights into morin as a potential therapeutic agent against endometritis caused by LPS.

Conflict of interest: None of the authors have any conflict of interest to declare.

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Authors contribution: RWS, RS, and SCH: Conceived and designed the study, as well as the visualization, and supervision. AS, MA, SH, and MH: Executed Experiments. MTA, MM, and IS: Analysed the data, Software, and Validation. RS and SAR writing – original draft and preparation. AS, IS writing – review and editing. All authors known and approved the final manuscript.

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