Article

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Combination of Doxycycline with Metronidazole Protects against Pyroptosis in Rats with Endometritis through the Modulation of *TLR4*/*NF-κB* Pathway

Qi Yao[1,*], Dongmei Lin[1], Yanyan Yang[2], Yuehong Zhuang[3]

[1]Gynecology, Fuzhou Second Hospital, 350007 Fuzhou, Fujian, China
[2]Experimental Animal Center, Fujian University of Traditional Chinese Medicine, 350122 Fuzhou, Fujian, China
[3]Human Anatomy and Histoembryology, Fujian Medical University, 350000 Fuzhou, Fujian, China
*Correspondence: YaoQixmdxfzey@163.com (Qi Yao)
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Background: Endometritis is a condition usually resulted from the bacterial infection of uterus, causing pelvic disease, sepsis, shock, uterine necrosis and even death if it is inappropriately treated. The aim of this study is to explore the pathogenesis of endometritis, and investigate whether the combination of doxycycline and metronidazole offers stronger protection against lipopolysaccharide (LPS)-induced endometritis, and decipher more about the mechanisms underlying endometritis-related pyroptosis.

Methods: Sprague-Dawley (SD) rats were divided into five groups (n = 8 per group): control, model, metronidazole, doxycycline, and combination groups. In control group, the rats were injected with saline, while in other groups, lipopolysaccharide was injected into uterus of the rats to establish endometritis. Hematoxylin-eosin (H&E) staining was performed as part of the histopathological examination of endometrium. The integrity of chromatin and pyroptosis were evaluated by terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay. Western blot and quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) were performed to ascertain the activation of toll-like receptors (*TLR4*)/nuclear factor-kappa B (*NF-κB*) pathway by detecting protein levels of phosphorylated p50 (p-p50)/p50, phosphorylated nuclear factor-kappa B (p-NF-κB)/NF-κB, phosphorylated IkappaB (p-IκB), and TLR4 protein and mRNA. Development of pyroptosis was also detected by determining the levels of caspase-1 and caspase-5 through Western blot and qRT-PCR. Enzyme-linked immunosorbent assay (ELISA) was used to detect levels of interleukin (IL)-1β, IL-18, IL-2, IL-4, IL-6 and tumor necrosis factor alpha (TNF-α), and flow cytometry was adopted to determine T-helper (Th)1 and Th2 cell percentage to assess the extent of pyroptosis and Th1/Th2 imbalance.

Results: The uterine of the model group exhibited pathological alterations and higher degree of cell apoptosis. Compared with the control rats, model group showed lower protein levels of p-p50/p50 ($p < 0.001$), p-NF-κB/NF-κB ($p < 0.001$), p-IκB ($p < 0.001$), and TLR4 protein ($p < 0.001$) and mRNA ($p < 0.001$). Elevated levels of caspase-1 ($p < 0.001$), caspase-5 ($p < 0.001$), IL-1β ($p < 0.001$), IL-18 ($p < 0.001$), IL-2 ($p < 0.01$), TNF-α ($p < 0.05$) and Th1/Th2 ($p < 0.001$) as well as reduced levels of IL-4 ($p < 0.05$) and IL-6 ($p < 0.01$) were observed in the model group, which could however be reversed by metronidazole ($p < 0.01$) or doxycycline ($p < 0.01$), with a more significant effect detected if a combination of the two drugs was administered ($p < 0.01$).

Conclusions: The combination of doxycycline and metronidazole protects against rat endometritis by inhibiting *TLR4*/*NF-κB* pathway-mediated inflammation and suppressing pyroptosis.

Keywords: endometritis; *TLR4*; *NF-κB*; doxycycline; metronidazole; pyroptosis

Introduction

Endometritis manifests in two forms, namely acute endometritis and chronic endometritis, and is caused by bacterial infection of the uterus [1]. Usually, endometritis occurs upon the damage of endometrium. Endometritis can be caused by a variety of factors, including infection caused by improper pelvic surgery [2] and fertility-related risk factors [3]. The symptoms of endometritis include abdominal distending pain, abnormal vaginal bleeding or secretions, fever, lower abdominal or pelvic pain (uterine pain) [2]. The clinical manifestations rarely appear in the case of chronic endometritis; even if the symptoms manifest and become apparent, the condition could be misdiagnosed [4–6]. At present, oral antibiotics stand as the chief treatment for endometritis [4,7,8], including doxycycline, metronidazole, and ofloxacin. Several studies have shown that incomplete abortion or uterine involution could lead to infertility [9] and pelvic disease [10]. These complications could further result in sepsis [11], shock [12], uterine necrosis [13] and even death [7]. Therefore, it is imperative to search for a more efficient treatment for endometritis.

Table 1. Animal groups and treatments.

Group (n = 5)	Treatment
Control	Injection of 1 mg/kg saline into the uterus
Model	Injection of 1 mg/kg of 1 mg/mL LPS into the uterus [20]
Metronidazole	Injection of 1 mg/kg of 1 mg/mL LPS into the uterus, and intraperitoneal injection of 135 mg/kg/day metronidazole for 5 consecutive days [21]
Doxycycline	Injection of 1 mg/kg of 1 mg/mL LPS into the uterus, and intraperitoneal injection of 100 mg/kg/day doxycycline for 5 consecutive days [22]
Combination	Injection of 1 mg/kg of 1 mg/mL LPS into the uterus, and intraperitoneal injection of 135 mg/kg/day metronidazole + 100 mg/kg/day doxycycline for 5 consecutive days

LPS, lipopolysaccharide.

Treatment involving appropriate antibiotics can limit the plasma cells from infiltrating the endometrial stroma. In general, doxycycline is employed as the first-line treatment, and metronidazole as the second course of antibiotic treatment. However, this treatment plan is only effective in patients who are not resistant to doxycycline [14]. Related studies [15,16] have shown that the combined use of ceftriaxone, doxycycline and metronidazole in the treatment of acute pelvic inflammatory disease is significantly more effective than when these antibiotics were used individually. Pyroptosis has been proved to be related to the progression of endometritis in many animal models [17–19], but the underlying mechanism remains largely uncharacterized.

The objective of this study was to investigate whether the combination of doxycycline and metronidazole can regulate pyroptosis in rats with endometritis through toll-like receptors (*TLR4*)/nuclear factor-kappa B (*NF-κB*) pathway. We hypothesized that the combination of drugs is more effective at inhibiting pyroptosis in rats with endometritis. To test the hypothesis, a series of experiments dedicated to examining the pathological changes as well as characterizing the profile of related proteins and inflammatory factors in rats with endometritis were performed after antibiotics administration.

Methods

Establishment of Endometritis Rat Model

All animal experiments described in this paper were approved by the Ethics Committee of Fuzhou Second Hospital of Xiamen University (Approval No. 20210315). A total of 40 female Sprague-Dawley (SD) rats (9–10 weeks old, 180–200 g), purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China), were housed in an environment set at a room temperature of 25 °C and a humidity of 50–68%. The animals were given free access to food and water.

The rats were divided into 5 groups, with 8 rats per group. All rats were anesthetized using sodium pentobarbital (50 mg/kg, intraperitoneal injection). A pipette gun tip (1 mL), whose sharp part was cut off, was used as a dilator of rat vagina, which was inserted into the rat vulva. A blunt needle, which was mounted to a syringe, was inserted into the rat uterus. Afterward, 1 mg/kg of lipopolysaccharide (LPS; dissolved in saline at concentration of 1 mg/mL; cat no. HY-D1056-50217, MedChemExpress, Monmouth Junction, NJ, USA) was injected into the uterus to complete the process of establishing endometritis rat model. No rats died from the construction of endometritis. After a 24-hour observation, 15 rats, with 3 rats from each group, were sacrificed by means of cervical dislocation after being anesthetized, and the collected uterine tissues were examined to confirm whether endometritis was successfully induced in the rats. Metronidazole (HY-B0318) and doxycycline (HY-N0565) were purchased from MedChemExpress (Monmouth Junction, NJ, USA). Grouping of rats and the exact treatments given to every group are presented in Table 1 (Ref. [20–22]). After treatments, the remaining total 25 rats (5 rats per group) were euthanized by cervical dislocation after being anesthetized, and uterus was extracted from each rat, fixed for 24 h in 4% paraformaldehyde, dehydrated and embedded in paraffin, and cut into 8-µm-thick sections. The blood samples of the rats were collected in blood tubes (BD Vacutainer; BD Bioscience, San Jose, CA, USA).

Hematoxylin-Eosin (H&E) Staining

Paraffin sections were placed in a series of xylene (10023418, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and ethanol (u1012772, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) solutions for dewaxing, followed by hematoxylin staining of the nucleus and eosin staining of the cytoplasm (C0109, Beyotime, Shanghai, China). Finally, the neutral gum (N116470, Aladdin, Shanghai, China) was used for sealing after dehydration. The slides were observed under a microscope (CK31, OLYMPUS, Tokyo, Japan), and the captured images were analyzed using an imaging system (TVO.63XC-MO, GUANGZHOU MSHOT PHOTOELECTRIC TECHNOLOGY Co., Ltd., Guangdong, China) and a digital full-scan instrument (PANNORAMIC SCAN, 3DHISTECH Co., Ltd., Budapest, Hungary).

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Assay

After being homogenized using the homogenizer (CN-41056-98, Cole-Parmer Instrument Company, LLC., Shanghai, China), uterine tissue homogenate was subjected to total RNA extraction using RNA Simple Total RNA Kit (DP424, Tiangen Biotech Co., Ltd., Beijing, China). Reverse transcription of the extracted RNA was performed using the FastQuant cDNA Synthesis Kit (KR116, Tiangen Biotech Co., Ltd., Beijing, China). All measurements were analyzed by $2^{-\Delta\Delta CT}$ method. The primer sequences were as follows: *TLR4* (forward 5′-AATCTGGTGGCTGTGGAG-3′, reverse 5′-CCCTGAAAGGCTTGGTCT-3′) and *β-actin* (forward 5′-GGCTGTATTCCCCTCCATCG-3′, reverse 5′-CCAGTTGGTAACAATGCCATGT-3′).

Western Blot

Proteins were extracted from collected tissues using the cell lysis buffer (P0013J, Beyotime, Shanghai, China) and their concentrations were determined using the enhanced bicinchoninic acid (BCA) protein assay kit (P0010S, Beyotime, Shanghai, China). The proteins were incubated with primary antibodies overnight at 4 °C; the primary antibodies include phosphorylated p50 (p-p50; 1:1000 dilution, cat. no. 710460, Invitrogen, Carlsbad, CA, USA), p50 (1:1000 dilution, cat. no. 51-3500, Invitrogen, Carlsbad, CA, USA), phosphorylated nuclear factor-kappa B (p-NF-κB; 1:1000 dilution, cat. no. 3033, Cell Signaling Technology, Boston, MA, USA), NF-κB (1:1000 dilution, cat. no. 710048, Invitrogen, Carlsbad, CA, USA), phosphorylated IkappaB (p-IκB; 1:1000 dilution, cat. no. 2859, Cell Signaling Technology, Boston, MA, USA), TLR4 (1:1000 dilution, cat. no. 66350-1-lg, Proteintech, Wuhan, China), caspase-1 (1:1000 dilution, cat. no. 22915-1-AP, Proteintech, Wuhan, China), caspase-5 (1:1000 dilution, cat. no. ET1612, HuaBio, Hangzhou, China), and β-actin (1:1000 dilution, cat. no. TA-09, ZSGB-Bio, Beijing, China). Horseradish peroxidase (HRP)-conjugated goat anti-mouse/rabbit IgG (1:2000 dilution, cat. no. ZB-2305/ZB-2301, ZSGB-Bio, Beijing, China) was used as secondary antibody in this experiment. The protein bands were then visualized with the aid of enhanced chemiluminescence kit (E411-04, Vazyme Biotech Co., Ltd., Nanjing, China) and analyzed using ImageJ software (Fiji software version 2.14.0, LOCI, University of Wisconsin, Madison, WI, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

To prepare the tissue homogenate for the ELISA experiment, 0.1 g of uterine tissue was mixed with 500 µL of RIPA buffer (P0013E, Beyotime, Shanghai, China) containing 1% protease inhibitor (HY-17541, MedChemExpress, Monmouth, NJ, USA), and then, the tissue-buffer mixture was homogenized on ice using a tissue homogenizer (CN-41056-98, Cole-Parmer Instrument Company, LLC., Shanghai, China). The homogenate was then centrifuged at 1500 ×*g* and 4 °C for 10 min. The supernatant was collected for the assay or stored at –20 °C. Levels of interleukin (IL)-1β, IL-18, IL-2, IL-4, IL-6 and tumor necrosis factor alpha (TNF-α) were detected by ELISA kits purchased from Wuhan Colorful Gene Biotech Co., Ltd. (Wuhan, China): IL-1β (JYM0419Ra), IL-18 (JYM0650Ra), IL-2 (JYM0042Ra), IL-4 (JYM1265Ra), IL-6 (JYM0646Ra), and TNF-α (JYM0419Ra). A microplate reader (MOLECULAR DEVICES, CMax Plus, Shanghai, China) was used to read the absorbance level at 450 nm.

Apoptosis Assay

Cell apoptosis in tissue sections were determined by terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) Kit (G1501, Wuhan Servicebio Technology Co., Ltd., Wuhan, China) in adherence to the manufacturer's instruction. After staining the tissue sections with DAPI (C1002, Beyotime, Shanghai, China), the slides were observed under an inverted fluorescence microscope (Nikon, Tokyo, Japan).

Flow Cytometry

To derive peripheral blood mononuclear cells (PBMCs), the collected blood samples were subjected to centrifugation (Ficoll Paque; Amersham Biosciences AB, Uppsala, Sweden) at 400 ×*g* and 18 °C for 10 min. After separation, the PBMCs were washed with phosphate-buffered saline (PBS) and then suspended in RPMI-1640 medium (iCell-0002, iCell Bioscience Inc. Shanghai, China). The prepared cell suspensions (100 µL) were stained with 5 µL of *CD4* APC (551980, BD Biosciences, San Jose, CA, USA) at 4 °C for 20 min and 20 µL of *IFN-γ* PE (559499, BD Biosciences, San Jose, CA, USA) for Th1 determination or 5 µL of *IL-4* PE-Cy7 (560699, BD Biosciences, San Jose, CA, USA) for Th2 determination for 30 min at 4 °C and away from the light. The samples were analyzed on BD FACSCantoII flow cytometer (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

The data are expressed as mean ± standard deviation, and comparative analyses between groups, such as Student's *t*-test or one-way analysis of variance (ANOVA) with Turkey's post hoc test, were performed using GraphPad 8.0.2 software (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

Results

Pathological Findings of Rat Uterus and Expression of TLR4/NF-κB Pathway-Related Protein

H&E staining revealed that the occurrence of epithelial cell degeneration and cytoplasmic vacuolization in the

mucosal layer of rats in the control group. There was a gap between the mucosal epithelium and the lamina propria. In the lamina propria, numerous uterine glands appeared to be slightly dilated, accompanied by mild lymphocyte infiltration. On the other hand, apoptosis, along with nuclear fragmentation, was found to occur in many mucosal epithelial cells of the model group. Accompanied by a slight uterine glandular dilatation, the lamina propria showed diffuse infiltration of lymphocytes and neutrophils, and dilatation of uterine glands (Fig. 1A). Based on the Western blot results, the levels of p-p50/p50 (Fig. 1B; $p < 0.001$), p-NF-κB/NF-κB (Fig. 1B; $p < 0.001$), p-IκB (Fig. 1C; $p < 0.001$) and TLR4 (Fig. 1D; $p < 0.001$) in model group were higher than those in control group. Moreover, a higher expression of *TLR4* mRNA was noted in model group than in control group (Fig. 1E; $p < 0.001$). These findings underlined the successful establishment of the endometritis rat model (model group), which was characterized by the more pronounced activation of the *TLR4/NF-κB* pathway.

Effect of Antibiotics Treatment on the Expression of TLR4/NF-κB Pathway Proteins

The expression levels of *TLR4/NF-κB* pathway-related proteins such as p-p50/p50 (Fig. 2C; $p < 0.001$), p-NF-κB/NF-κB (Fig. 2B; $p < 0.001$), p-IκB (Fig. 2D; $p < 0.001$) and TLR4 (Fig. 2E; $p < 0.001$), as well as expression level of *TLR4* mRNA (Fig. 2E; $p < 0.001$) were lower in metronidazole or doxycycline group than in model group, with further reductions observed in combination group (Fig. 2A–E, $p < 0.001$) as compared with metronidazole or doxycycline group. These findings indicated that the antibiotics could suppress the activation of *TLR4/NF-κB* pathway, and the combination of metronidazole and doxycycline presented the strongest inhibitive impact on the pathway.

Effect of Antibiotics Treatment on Pyroptosis of Rat Uterine Cells

After H&E staining, we observed a small extent of apoptosis in the mucosal epithelial cells and uterine glandular epithelial cells. Nuclear fragmentation or loss of epithelial structure were observed in the mucosal layer of the rats in metronidazole group. We also found that the uterine glands in the lamina propria decreased in number, and histologically, they appeared degenerated and were exposed. A small number of uterine glands dilated and uterine gland epithelial cells appeared degenerated. In the metronidazole group, cytoplasmic vacuolization was observed, along with the scattered distribution of lymphocytes and neutrophils.

A small extent of epithelial cell degeneration and cytoplasmic vacuolization were observed in the mucosal layer of the rats in doxycycline group. The number of glands in the lamina propria was significantly reduced, and traces of lymphocytes and neutrophils were observed.

Similar to the doxycycline group, epithelial cell degeneration and cytoplasmic vacuolization were observed in the mucosal layer in the rats of combination group. Dilatation of uterine glands was detected in the lamina propria (Fig. 3A). The representative image of TUNEL showed that both metronidazole group and doxycycline group exhibited lower level of apoptosis, as compared with model group, and the apoptosis level of combination group was lower than that of metronidazole group or doxycycline group (Fig. 3B). Caspase-1 protein (Fig. 3C,D; $p < 0.001$) and mRNA (Fig. 3D; $p < 0.001$) levels in the model group were higher than those in the control group, and the same parameters in the combination group were lower than those in the model group (Fig. 3C,D; $p < 0.001$). The levels of caspase-5 protein (Fig. 3C,E; $p < 0.001$) and mRNA (Fig. 3E; $p < 0.001$) in the model group surpassed those in the control group, and the levels of caspase-5 protein and mRNA in the metronidazole group (Fig. 3C,E; $p < 0.01$) and the combination group (Fig. 3C,E; $p < 0.001$) were lower than those in the model group.

According to the ELISA results, the levels of IL-1β (Fig. 3F; $p < 0.001$) and IL-18 (Fig. 3G; $p < 0.001$) in the model group were higher than those in the control group. Reduction in the levels of these interleukins was observed in the metronidazole group ($p < 0.001$) and doxycycline group ($p < 0.001$), with more pronounced decrease detected in the combination group ($p < 0.001$). Altogether, these findings suggested the synergistic effect of the combination of metronidazole and doxycycline on the suppression of endometritis-related pyroptosis.

Effect of Antibiotics Treatment on Th1/Th2 Imbalance

According to the ELISA results, the concentration of TNF-α (Fig. 4A; $p < 0.001$) and IL-2 (Fig. 4B; $p < 0.001$) was higher in the model group than in the control group. Significant decrease in the concentration of these pro-inflammatory factors was observed in the metronidazole group ($p < 0.01$) and doxycycline group ($p < 0.01$), and further reduction was noted in the combination group ($p < 0.01$). The concentration of IL-4 (Fig. 4C; $p < 0.05$) and IL-6 (Fig. 4D; $p < 0.01$) was lower in the model group compared with the control group; both metronidazole group ($p < 0.01$) and doxycycline group ($p < 0.001$) showed increased concentration of these interleukins, with more pronounced elevation in the combination group ($p < 0.001$). Fig. 4E depicts that the Th1/Th2 was increased in the rats of the model group ($p < 0.001$), but decreased in the metronidazole group ($p < 0.001$) and doxycycline group ($p < 0.001$) and further reduced in the combination group ($p < 0.001$). These results indicate that the Th1/Th2 in the model group, metronidazole group and doxycycline group were in the state of imbalance. The noteworthy finding from this set of results is that the Th1/Th2 balance was shifted toward Th2 in the combination group.

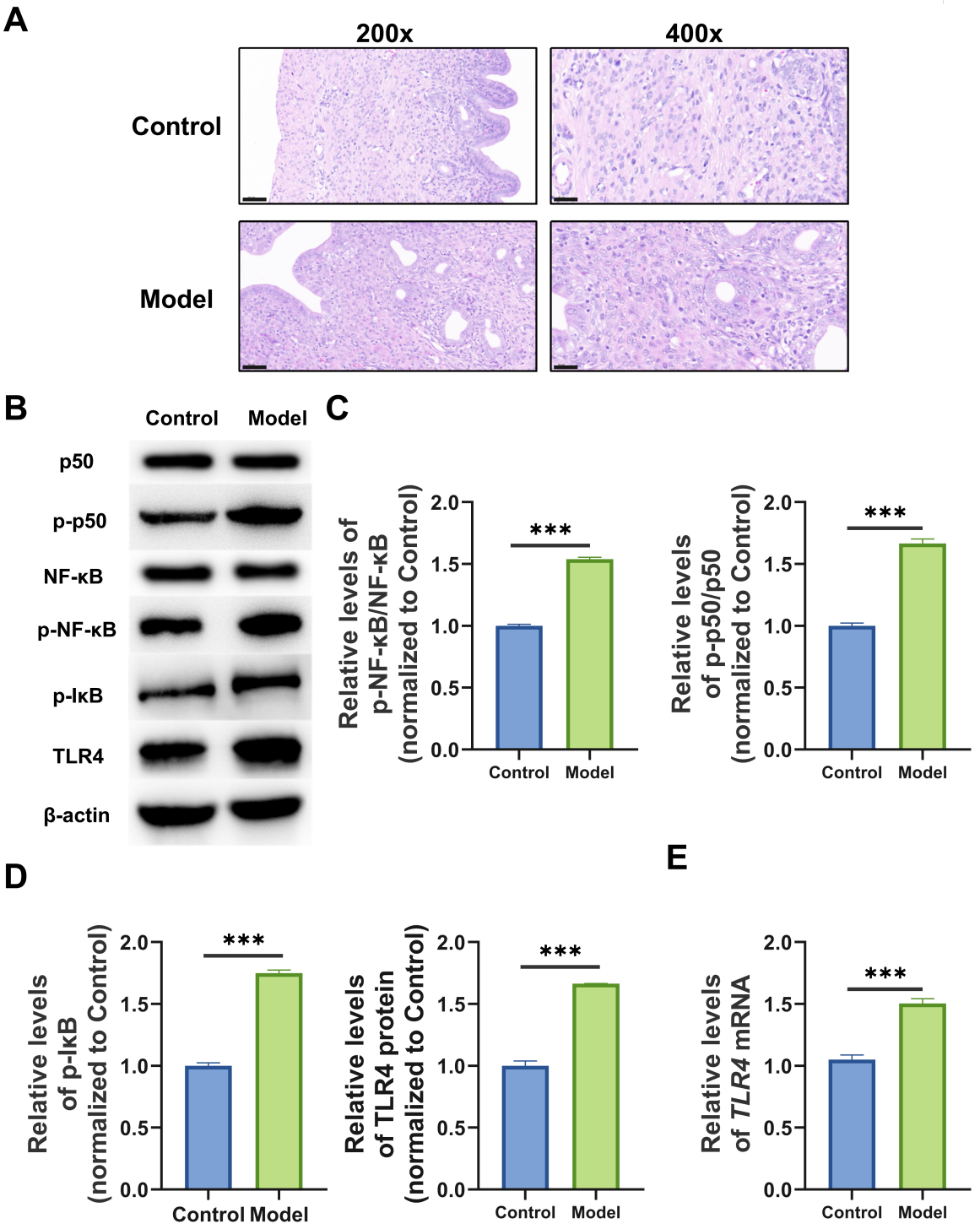

Fig. 1. Histopathological findings of rat uterus and expression of toll-like receptors 4 (*TLR4*)/nuclear factor-kappa B (*NF-κB*) pathway-related proteins. (A) Hematoxylin-eosin (H&E) staining of uterine. Scale bar: 50 μm (200×), 20 μm (400×). (B) Representative images of Western blot. (C) Relative protein levels of phosphorylated p50 (p-p50)/p50 and phosphorylated nuclear factor-kappa B (p-NF-κB)/NF-κB. (D) Relative protein levels of phosphorylated IkappaB (p-IκB) and TLR4. (E) Relative mRNA expression level of *TLR4*. n = 5. ***$p < 0.001$.

Discussion

Aside from being an important indicator of female health, healthy endometrium also serves as a prerequisite for ensuring normal fertility. Therefore, it is crucial to administer antibiotics for treating endometritis in early stage [23], as they can greatly alleviate the long-term impact of endometritis on the reproductive health of patients. Doxy-

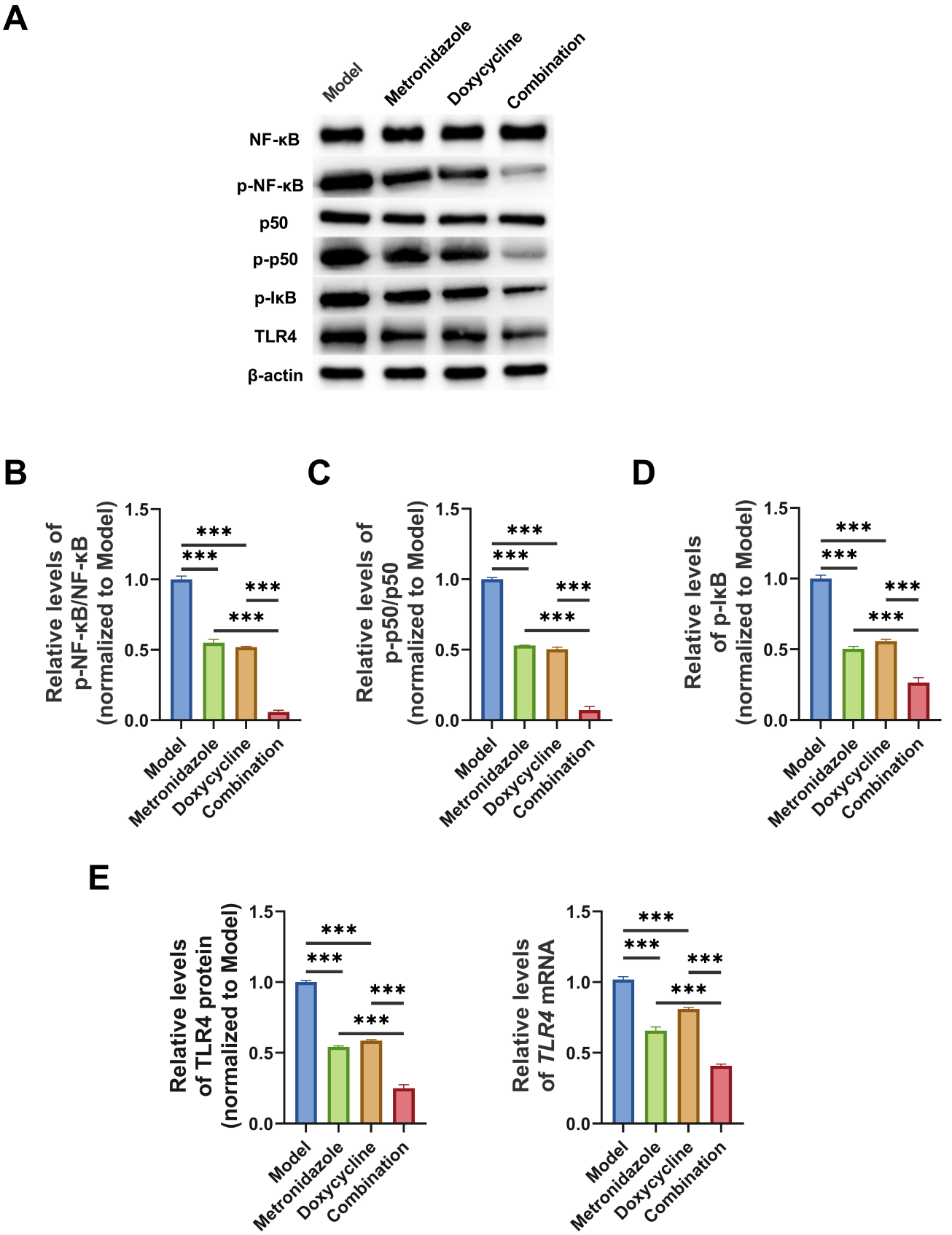

Fig. 2. Effect of antibiotics treatment on the expression of *TLR4*/*NF-κB* pathway proteins. (A) Representative Western blot images. Relative protein levels of (B) p-NF-κB/NF-κB, (C) p-p50/p50, and (D) p-IκB. (E) Relation expression levels of TLR4 protein and *TLR4* mRNA. n = 5, ***$p < 0.001$.

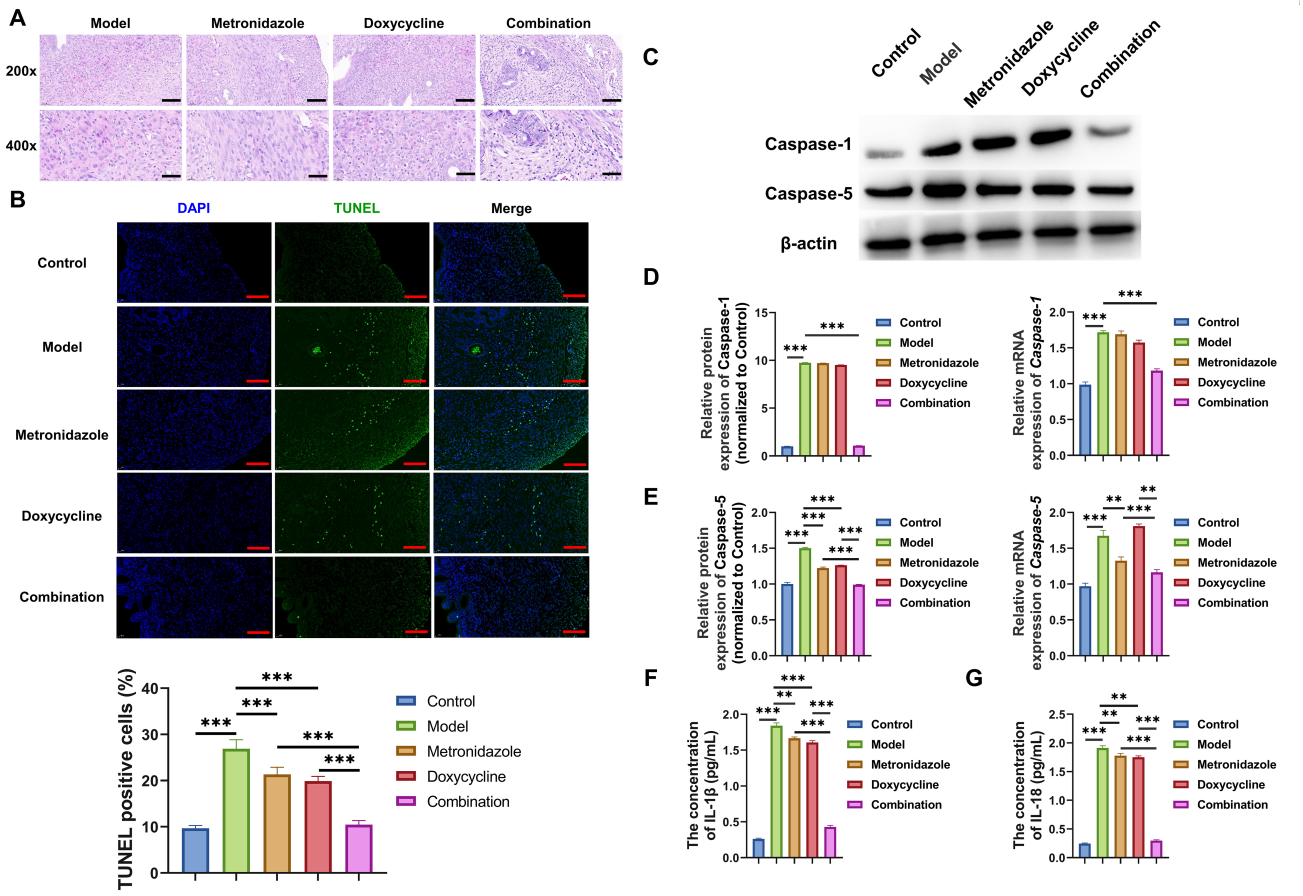

Fig. 3. Effect of antibiotics treatment on pyroptosis of rat uterine cells. (A) H&E staining of uterine; scale bar: 50 μm (200×), 20 μm (400×). (B) Apoptosis visualized with the aid of terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay; scale bar: 50 μm. (C) Representative Western blot of different groups. (D) Relative expression level of caspase-1. (E) Relative expression level of caspase-5. (F) Concentration of interleukin (IL)-1β. (G) Concentration of IL-18. n = 5, $^{**}p < 0.01$, $^{***}p < 0.001$.

cycline is a semi-synthetic tetracycline derivative that can defend against both aerobic and anaerobic bacteria [24,25]. Metronidazole should always be used in combination with a drug that is effective against aerobic or facultative anaerobic bacteria [26–28]. It has been shown that the efficacy of the combined use of antibiotics exceeds that of single antibiotic, because the combination approach presents a range of antibacterial drugs against different strains of bacteria [29]. This idea was corroborated in the present study, which also attempted to investigate whether the pyroptosis can be attenuated by the antibiotics and how *TLR4/NF-κB* pathway played roles in it.

We first detected the histopathological features of uterine tissue sections in control group and model group by means of H&E staining. The model group showed conspicuous pathological features of endometritis, mucosal epithelial cell apoptosis, nuclear fragmentation, and diffuse infiltration of lymphocytes and neutrophils into lamina propria. These histological results strongly indicated that the endometritis rat model was successfully constructed, and a microscopic examination of the H&E-stained tissue sections revealed the occurrence of pyroptosis [30,31]. The diffuse infiltration of lymphocytes and neutrophils sheds light on a potential link between endometritis and pyroptosis. In order to further verify the relationship between *TLR4/NF-κB* signaling pathway and endometritis in rats, we evaluated the expression of the pathway-related proteins in endometrial tissues. The expression studies provided a clearer picture that the occurrence and development of endometritis is accompanied by an alteration to the expression of proteins associated with *TLR4/NF-κB* pathway, suggesting that endometritis is regulated by *TLR4/NF-κB* pathway. Moreover, our results showed that the expression of pathway-related proteins in the combination group was significantly lower than that in the model group, and its effect on protecting against pyroptosis in uterus of endometritis was better than that in doxycycline group and metronidazole group. At the same time, the symptoms of endometritis were mitigated in the combination group.

To further explore the effect of antibiotics on endometritis and the associated effect on pyroptosis, we assessed the apoptosis of endometrial tissue and measured the expression levels of caspase-1, caspase-5, IL-1β and IL-18. The results showed that pyroptosis occurred in endometri-

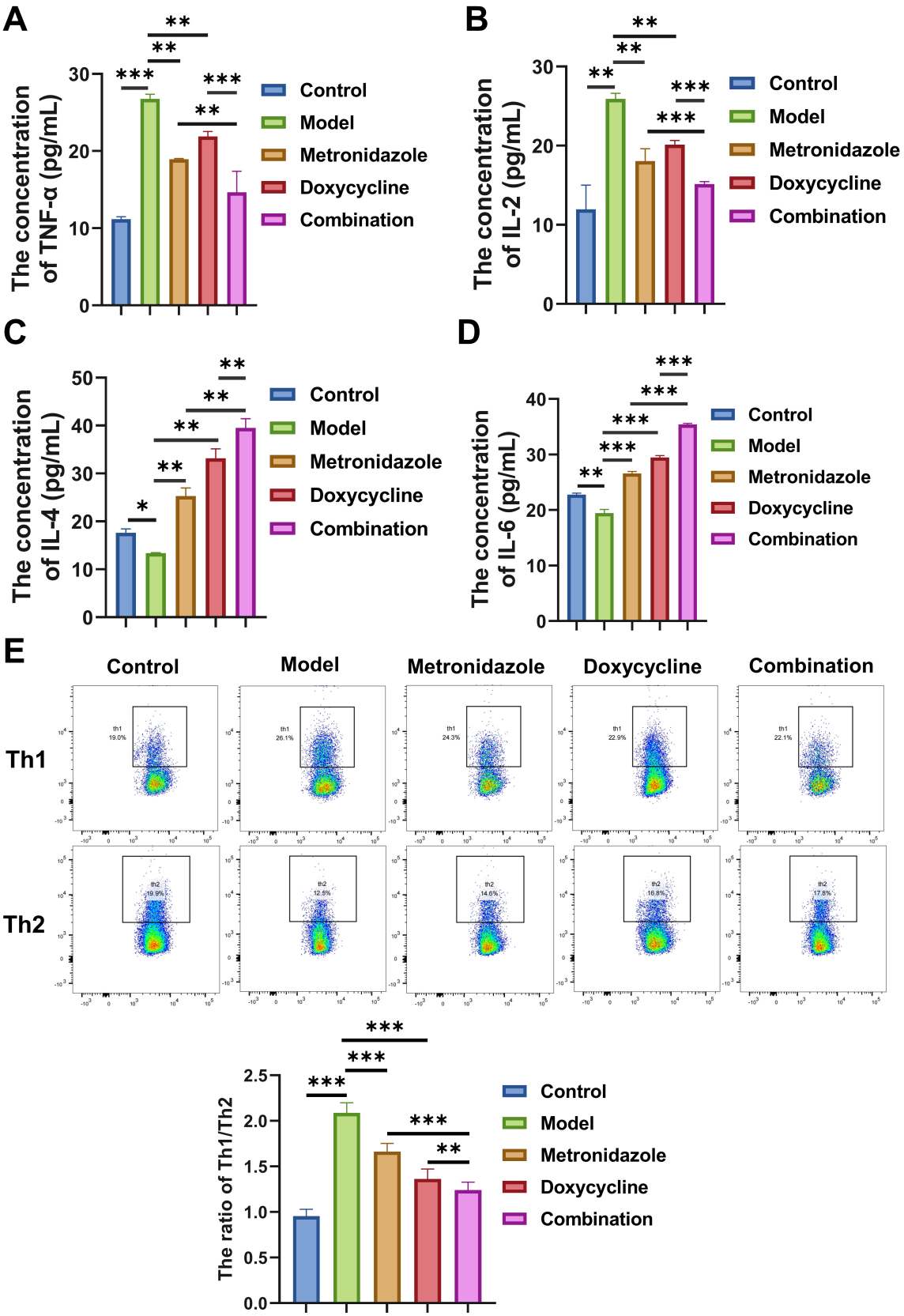

Fig. 4. Effect of antibiotics treatment on Th1/Th2 imbalance. (A–D) The bar charts show the concentrations of (A) Tumor necrosis factor alpha (TNF-α), (B) IL-2, (C) IL-4, and (D) IL-6 of different groups. (E) Representative results of flow cytometry and quantification of Th1/Th2 ratio. n = 5, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

tis tissues, which could be inhibited by the use of doxycycline and metronidazole. The inhibitory effect of combination group was better than that of doxycycline group and metronidazole group. A relevant study [32] has shown that a shift of Th1/Th2 towards the Th2 direction can significantly reduce local inflammation in rats with chronic endometritis. Our results also revealed that the Th1/Th2 balance in the doxycycline group and the metronidazole group shifted in the direction of Th2, and a higher degree of inclination in terms of the shift towards Th2 was found in the combination group. Taken together, the rational combination of antibiotics can offer an avenue to better inhibit pyroptosis and alleviate inflammation. The advantages of using a combination of antibiotics in the treatment of endometritis are similar to the same kind of treatment for other diseases. The merit of the current study lies in filling the knowledge gap regarding the combined use of doxycycline and metronidazole in the treatment of endometritis.

Several ways that can be adopted to address the shortcomings of this study should be acknowledged. Firstly, more studies should be performed to identify the optimal concentration of drug to be administered for acquiring the maximum therapeutic effect of doxycycline and metronidazole while controlling their toxicities. Additionally, more efforts should be made to explore pyroptosis, in terms of especially the relation of pyroptosis with gasdermin D (GSDMD) expression and the morphology of pyroptotic cells using transmission electron microscope; we believe these findings derived from these studies would provide a clearer understanding of endometritis and the treatment based on doxycycline and metronidazole. Moreover, clinical data regarding the treatment of endometritis, such as combined antibiotics, should be collected for further validation purposes.

Conclusions

In summary, the combination of doxycycline and metronidazole provide a more effective avenue for treating endometritis. Additionally, this approach can suppress endometrial cell pyroptosis and *TLR4*/*NF-κB* pathway activation so as to slow down progression and deterioration of endometritis while aiding in treatment acceleration.

Availability of Data and Materials

Data to support the findings of this study are available on reasonable request from the corresponding author.

Author Contributions

QY, DML and YYY contributed to the concept and designed the research study. DML and YHZ performed the research. YYY provided help and advice on the experiments. QY and YHZ contributed to the analysis and interpretation of the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All animal experiments described in this paper were approved by the Ethics Committee of Fuzhou Second Hospital of Xiamen University (Approval No. 20210315).

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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