

# Effects of salpingitis simulation on the morphology and expression of inflammatory-related genes of oviduct in laying hens

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**ABSTRACT** This study was conducted to simulate salpingitis of laying hens by observing the morphology and expression of inflammatory genes in the oviduct. A total of one hundred twenty 81-wk-old Roman Pink laying hens in good physical condition without the oviduct disease with an average egg production rate of 76% were fed a basal diet for 2 wks and then randomly allocated into 4 groups (6 replicates/group, 5 birds/replicate). The experimental treatments were as follows: 1) Control group (treated with PBS); 2) Organic chemical reagent (OCR) group; 3) Lipopolysaccharide (LPS) group; 4) LPS + OCR group. First, the chickens were kept upside down to make ectropion and exposure of the apertura uterinae; then prepared reagents were poured into the uterine part of the fallopian tube by using the chicken vas deferens (1 mL/layer); finally, the chickens were kept in the inverted position for 5 to 10 min. The fallopian tube samples (the magnum, isthmus, and uterus) were collected after 48 h of treatment. Compared

with the control, treatment with LPS+OCR decreased ( $P < 0.05$ ) the secondary villus length and primary villus area in magnum and villus length in isthmus ( $P < 0.05$ ). An increase ( $P < 0.05$ ) of the intervillous space of uterus was observed in LPS + OCR group compared with the control. The expressions of interleukin-6 mRNA of magnum and interferon- $\gamma$  (IFN- $\gamma$ ) of isthmus in the LPS and LPS+OCR treatments were higher ( $P < 0.05$ ) than that in control. Compared with the control, treatment with LPS+OCR increased ( $P < 0.05$ ) the expressions of IFN- $\gamma$  mRNA of magnum and IFN- $\gamma$ , tumor necrosis factor- $\alpha$  and inducible nitric oxide synthase mRNA of uterus in laying hens. In conclusion, the results of morphological damage of fallopian tube tissue and increased expression of inflammatory factors in LPS + OCR treatment group suggested that LPS+OCR treatment can provide data basis to establish salpingitis model in laying hens for studying the pathogenesis of it.

**Key words:** salpingitis, lipopolysaccharide, inflammatory-related cytokines, histomorphometry, laying hen

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## INTRODUCTION

Under the mode of large-scale intensive farming, laying hens are in sub-health state for a long time, which is easy to induce a common disease, salpingitis of laying hens (Wang et al., 2020). The main clinical characteristics of salpingitis of laying hens are decreased egg production, no or short maintenance time of peak of laying eggs, poor eggshell quality, increase of thin, soft and sandy shell eggs, which causes great economic losses to the poultry industry. The success of the poultry industry depends mainly on the reproductive status of the birds (Hanafy and Elnesr, 2021; El Sabry et al., 2022). So the

salpingitis is one of the frequent diseases threatening the healthy development of laying hens farming industry (Fang et al., 2021).

In salpingitis of laying hens, *Escherichia coli* and *Salmonella* belonging to Gram-negative bacteria are considered to be the common pathogenic bacteria of salpingitis of laying hens (Poulsen et al., 2020). Lipopolysaccharide (LPS) is a kind of endotoxin composed of macro-molecular lipids and polysaccharides on the outer membrane of Gram-negative bacteria by covalent bond (Lepper et al., 2002). When Gram-negative bacteria infect the fallopian tubes of laying hens, the secretion of pro-inflammatory cytokines increases dramatically under the stimulation of LPS released during bacterial death and reproduction (Wu et al., 2017), resulting in salpingitis.

As for salpingitis of laying hens, the common solution in production is to put health care drugs on a regular basis. From the perspective of food safety, eggs should not be eaten during the period of putting drugs and the

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withdrawal time, which will affect the economic benefits of laying hens. At present, many studies have been reported on the cell-to-whole effects and nutrition regulation of LPS (or *Escherichia coli*)-induced endometritis in mammals (Williams et al., 2008; Piras et al., 2017; Wu et al., 2018; Xu et al., 2020). However, there is no report on the establishment of salpingitis model of laying hens, which greatly delays the research progress of salpingitis in laying hens.

Thus, the main objective of this study was to simulate salpingitis of laying hens by observing the morphology and expression of inflammatory-related genes in the fallopian tube, so as to provide data support for establishing the model of salpingitis to study the pathogenesis of it.

## MATERIALS AND METHODS

All experimental procedures were conducted in accordance with Hubei Provincial Regulations for Laboratory Animals (011043145-029-2013-000009), and were approved by the Institutional Animal Care and Use Committee of Wuhan Polytechnic University (Number: WPU202205001).

### Birds, Diets and Management

A total of one hundred twenty 81-wk-old Roman Pink laying hens in good physical condition without the oviduct disease with an average egg production rate of 76% were fed a basal diet for 2 wks and then randomly allocated into 4 groups (6 replicates/group, 5 birds/replicate). The experimental treatments were as follows: 1) Control group (treated with PBS, I70011044); 2) organic chemical reagent (OCR) group (25% liquefied phenol (10015318) + 2.5% Tween 20 (30189328) + 2.5% Span 20 (30170428) + 10% glucose (63005518) + 60% PBS); 3) LPS group (LPS solution prepared by PBS; 1.85 mg/mL LPS; Sigma, SL263003); 4) LPS + OCR group (LPS: 3.7 mg/mL; OCR: the same as OCR group; LPS:OCR (v:v) = 1:1). Laying hens were fed the basal diet shown in Table 1 during the whole experiment. Layers (1 bird per cage) in each replicate were raised in 5 adjacent cages. The size of each cage (equipped with one nipple drinkers and one feeder) was 45 × 50 × 45 cm<sup>3</sup>. An enclosed, ventilated, and conventional room was controlled at 25°C by a daily lighting schedule of 16 h light and 8 h dark. Feed and water were offered ad libitum.

### Simulation of Salpingitis With Prepared Reagents

The experimental method was consistent with that reported by Fang et al. (2021). First, the chickens were kept upside down to make ectropion and exposure of the apertura uterinae; then prepared reagents were poured into the uterine part of the fallopian tube by using the chicken vas deferens (1 mL/layer); finally, the chickens

**Table 1.** The composition and nutrient levels of basal diet for laying hens.

Ingredients (%)	Content	Calculated nutrient levels (%)	
		Content	Content
Corn (7.8% CP)	20.00	ME (kcal/kg)	2713
Soybean meal (43% CP)	17.04	CP	15.30
Soybean oil	1.50	Calcium	4.00
Wheat	49.50	Total phosphate	0.53
Limestone (particle)	9.80	Available phosphorus	0.33
Dicalcium phosphate	1.25	Digestible lysine	0.63
Sodium chloride	0.15	Digestible methionine	0.31
Sodium hydrogen carbonate	0.25	Digestible threonine	0.43
L-Threonine	0.05		
L-Lysine (70%)	0.07		
Liquid DL-Methionine	0.10		
Choline chloride	0.15		
Vitamin premix <sup>1</sup>	0.02		
Trace mineral premix <sup>2</sup>	0.10		
Compound enzyme preparations	0.02		
Total	100		

<sup>1</sup>Provided per kilogram of diets: vitamin A 10000 IU, vitamin D<sub>3</sub> 2500 IU, vitamin E 26 mg, vitamin B<sub>1</sub> 2.0 mg, vitamin B<sub>2</sub> 6.0 mg, vitamin B<sub>6</sub> 3.0 mg, vitamin B<sub>12</sub> 0.025 mg, D-Biotin 0.050 mg, folic acid 1.0 mg, pantothenic acid 10 mg, niacin acid 30 mg.

<sup>2</sup>Provided per kilogram of diets: Cu 5 mg, Fe 25 mg, Mn 100 mg, Zn 60 mg, I 0.5 mg, Se 0.2 mg.

were kept in the inverted position for 5 to 10 min. The mental state of laying hens was observed. The basic diet was still provided to the laying hens during simulating salpingitis.

### Sample Collection

Forty-eight hours after experiment treatment, one bird from each group was randomly selected and marked. After 12-h feed withdrawal (water was offered ad libitum), hens were sacrificed humanely by CO<sub>2</sub> asphyxiation to collect samples. Following slaughter, the magnum, isthmus, and uterus of the fallopian tube were quickly removed and flushed with sterile saline solution. Part of each section (1 cm) isolated was fixed in 4% paraformaldehyde and kept at 4°C for a microscopic assessment of salpingitis morphology. Another part of each section (1 cm) isolated was collected and placed in liquid nitrogen for cytokine analysis.

### Oviduct Morphology Analysis

The fallopian tube samples (the magnum, isthmus, and uterus) were first washed under flowing water, subsequently dehydrated in different concentrations of ethanol, then dipped in xylene, next dipped and embedded in paraffin, then cut into cross-sections of 5 μm thickness and finally stained with hematoxylin and eosin (Solarbio Science & Technology Co., Ltd, Beijing, China) for conventional morphological evaluation (Shen et al., 2014). Images of the various parts of the fallopian tube were acquired using a DM3000 microscope (Leica, Wetzlar, Germany) (50 ×). The primary villus length, secondary villus length and primary villus area in the magnum and villus length in the isthmus were measured. The

intervillous spaces in the uterus were averaged for 3 to 5 adjacent villi spaces (averaged from ten values between 2 adjacent villi) in each slice. The measurements of fallopian tube morphology were made using Image-Pro software (Media Cybernetics, Rockville, MD).

### RNA Isolation and Quantitative Real-Time PCR

The expressions of interleukin-6 (IL-6), interferon- $\gamma$  (IFN- $\gamma$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and inducible nitric oxide synthase (iNOS) mRNA in magnum, isthmus and uterus were determined on a real-time PCR system (ABI 7500; Applied Biosystems, Foster City, CA) following the protocol of SYBR Premix Ex Taq kit (Takara Biotechnology (Dalian) Co., Ltd.) as previously described (Guo et al., 2017; Guo et al., 2020). First, the total RNA was extracted from approximately 20 mg of samples using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The concentration and purity of total RNA were quantified by measuring its optical density at 260 and 280 nm with a NanoDrop, ND-2000 UV-VIS spectrophotometer (Thermo Scientific, Wilmington, DE). The agarose gel electrophoresis was used to verify RNA integrity. Reverse transcription was performed from 1  $\mu$ g total RNA using the PrimeScript RT reagent kit with gDNA Eraser (Takara Biotechnology (Dalian) Co., Ltd., Dalian, China) according to the manufacturer's instructions. The oligonucleotide primers are listed in Table 2. The PCR was performed with the following thermal procedure: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and annealing and extension temperature at 60°C for 34 s. There were 6 samples for each group, and each sample was performed in triplicate. The mRNA levels were standardized as the ratio to  $\beta$ -actin in arbitrary units by the  $2^{-\Delta\Delta C_t}$  method and the data were expressed as the relative values to the control group.

### Statistical Analyses

All experimental data were presented as means with SEM and analyzed statistically by one-way ANOVA

**Table 2.** Primers used for real-time quantitative fluorescence PCR analysis.

Gene	Primer (5'→3')		Accession no.
	Forward	Reverse	
IL-6	CAAGGTGACGG AGGAGGAC	TGGCGAGGA GGGATTCT	AJ309540
IFN- $\gamma$	AGCTGACGGTGGA CCTATTATT	GGCTTTGCGC TGGATTC	NM_205149
TNF- $\alpha$	GAGCGTTGAC TTGGCTGTC	AAGCAACAACC AGCTATGCAC	NM_204267
iNOS	CAGCTGATTG GGTGTGGAT	TTTCTTTGGCCT ACGGGTC	U46504
$\beta$ -actin	GAGAAATTTGT GCGTGACATCA	CCTGAACCTCTC ATTGCCA	NM_205518

Abbreviations: IFN- $\gamma$ , interferon- $\gamma$ ; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

using SPSS 20.0. A Turkey test was used to determine significant differences among means. Values of  $P < 0.05$  were considered significant. All the graphs were made using GraphPad Prism 5.01.

## RESULTS

### Observation of Oviduct Morphology

The morphology of fallopian tube in laying hens and measurements of magnum, isthmus, and uterus were shown in Figure 1. Compared with the control, treatment with LPS + OCR decreased ( $P < 0.05$ ) the secondary villus length and primary villus area but didn't affect ( $P > 0.05$ ) primary villus length in magnum (Figure 1B). Compared with the control, LPS and LPS + OCR groups had lower villus length in isthmus ( $P < 0.05$ ) (Figure 1C). An increase ( $P < 0.05$ ) of the intervillous space of uterus was observed in LPS + OCR group compared with the control (Figure 1D).

### Expression of Inflammatory Genes

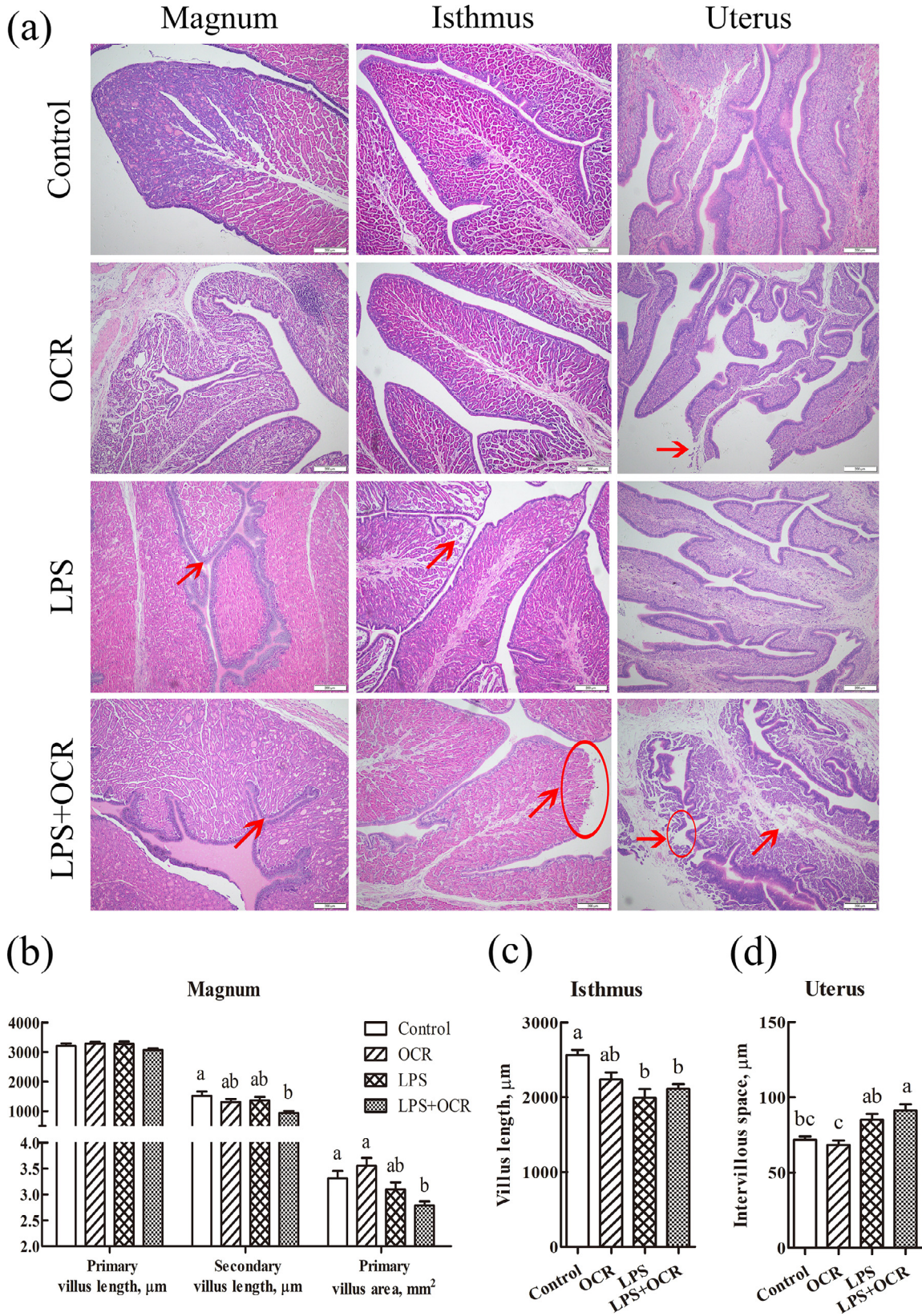
There was no difference ( $P > 0.05$ ) in the expressions of TNF- $\alpha$  mRNA in mamnum, IL-6, TNF- $\alpha$  and iNOS mRNA in isthmus in response to any reagent treatments (Figures 2–4). The mRNA expression of IL-6 in magnum and IFN- $\gamma$  in isthmus in the LPS and LPS + OCR treatments were higher ( $P < 0.05$ ) than that in control. Compared with the control, treatment with LPS + OCR increased ( $P < 0.05$ ) the mRNA expression of IFN- $\gamma$  in magnum and IFN- $\gamma$ , TNF- $\alpha$ , and iNOS in uterus of laying hens.

## DISCUSSION

At present, there were few reported methods for modeling salpingitis of laying hens. Pors et al. (2014) used laparotomy to inject 0.1 mL of *Escherichia coli* ( $8.6 \times 10^6$  CFU/mL) into the oviduct (5–7 cm away from the isthmus) and successfully made the model of salpingitis of laying hens. The advantage of this method is that the injection site is clear, but the application and promotion of this method in experimental research are limited by the difficulty of operation, high requirements for surgical environment, time-consuming and laborious operation, and limited number of molds made at the same time. Then open operation is very harmful to laying hens and prone to secondary systemic infection, resulting in increased mortality. The physiological indexes and production performance of laying hens vary greatly after successful modeling, which does not conform to the actual situation of salpingitis of laying hens in production. Therefore, it is not suitable to be used as a model to study the pathogenesis and the prevention and treatment effect of drugs.

Salpingitis model studies on other experimental animals such as rat and rabbit have been reported

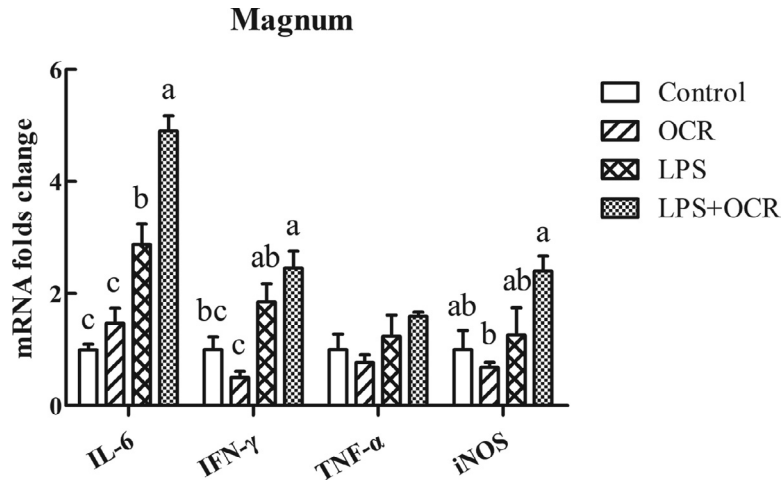




**Figure 1.** The morphology of fallopian tube in laying hens and measurements of primary villus length, secondary villus length and primary villus area in magnum, villus length in isthmus and intervillous space in uterus ( $50\times$ ). Data are presented as means and SEM. Values with no letters or the same superscript letters are not significantly different, whereas those with different superscript letters are significantly different ( $P < 0.05$ ). LPS, lipopolysaccharide; OCR, organic chemical reagent.

(Luo et al., 2015; Li et al., 2017; Liao et al., 2019). The more commonly used are methods of pathogenic microorganism modeling. This method is to inject mixed bacteria, *Escherichia coli*, *Staphylococcus*

*aureus* or *Chlamydia trachomatis* and other pathogenic microorganisms into the fallopian tube, so as to make animal salpingitis model. But the anatomical and physiological characteristics of the oviduct of



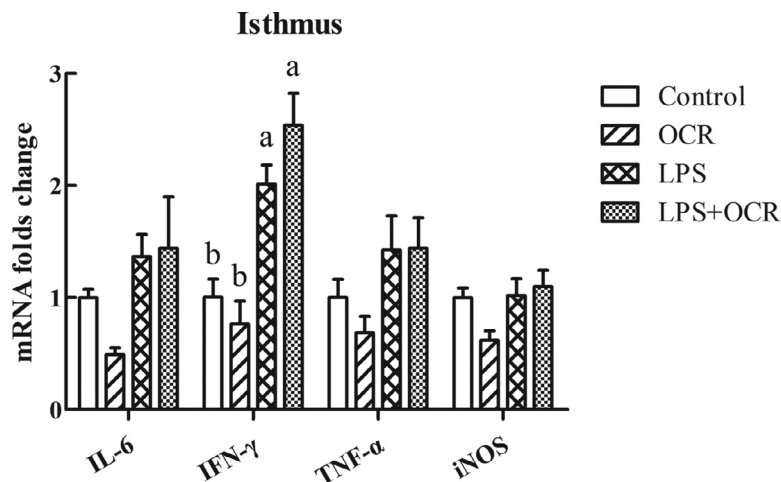
**Figure 2.** Expressions of IL-6, IFN- $\gamma$ , TNF- $\alpha$ , and iNOS mRNA in the magnum quantified with real-time PCR.  $\beta$ -Actin was used as a reference gene. Data are presented as means and SEM ( $n = 6$ ). Values with no letters or the same superscripts are not significantly different, whereas those with different superscript letters are significantly different ( $P < 0.05$ ). IL-6, interleukin-6; IFN- $\gamma$ , interferon- $\gamma$ ; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; OCR, organic chemical reagent.

laying hens are very different from those of rats, the cloaca of laying hens is the common opening of the digestive tract, reproductive tract and urinary tract. As a result, the oviduct of laying hens is exposed to microorganisms such as *Escherichia coli* in digestive tract for a long time, which has a natural resistance to pathogenic microorganisms and strong self-healing ability. Therefore, when this method is used to model salpingitis of laying hens, it often fails to produce typical salpingitis. Even when used in experimental animals such as rats, it also has the disadvantages of producing inflammation for a long time, mild symptoms, difficulty grasping accurate doses, and susceptibility to effects of microbial virulence and body immunity.

There is a modeling method, chemical reagent modeling method, which can quickly cause obvious inflammatory response with typical symptoms (Zhang et al.,

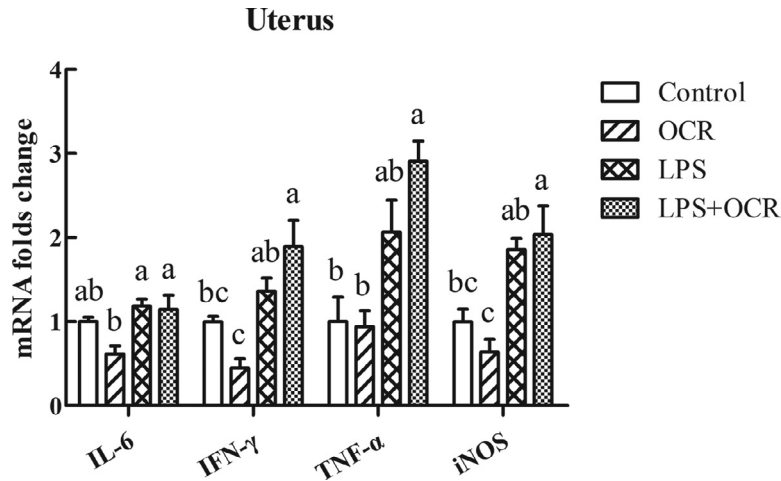
2018). Though the dose of chemical reagent is easy to grasp, the disadvantage is not completely consistent with the clinical pathogenesis of salpingitis. In the current study, there were no significant differences in the morphology and the expression of inflammation-related genes of fallopian tube between the OCR group and the control group. Therefore, the addition of OCR was not the main factor leading to salpingitis in laying hens. During the experiment, chemical modeling method and artificial sperm infusion method were combined to try to provide reproducible, obvious inflammatory characteristics, in line with the clinical practice of salpingitis simulation method for establishing the model of salpingitis in laying hens.

As a corrosive agent, liquefied phenol has the function of corroding fallopian tube mucosa and is the main component of chemical reagents (Song et al., 2017). Tween 20 (or tween 40), surface active agent, has solubilizing



**Figure 3.** Expressions of IL-6, IFN- $\gamma$ , TNF- $\alpha$ , and iNOS mRNA in the isthmus quantified with real-time PCR.  $\beta$ -Actin was used as a reference gene. Data are presented as means and SEM ( $n = 6$ ). Values with no letters or the same superscripts are not significantly different, whereas those with different superscript letters are significantly different ( $P < 0.05$ ). IFN- $\gamma$ , interferon- $\gamma$ ; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; OCR, organic chemical reagent; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .





**Figure 4.** Expressions of IL-6, IFN- $\gamma$ , TNF- $\alpha$ , and iNOS mRNA in the uterus quantified with real-time PCR.  $\beta$ -Actin was used as a reference gene. Data are presented as means and SEM ( $n = 6$ ). Values with no letters or the same superscripts are not significantly different, whereas those with different superscript letters are significantly different ( $P < 0.05$ ). IFN- $\gamma$ , interferon- $\gamma$ ; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; OCR, organic chemical reagent; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

effect (Eskandani et al., 2013), which can disperse phenol evenly and prevent excessive corrosion of local phenol concentration. Then span 20 (or span 40), surface active agent, has solubilizing effect, which can adjust the hydrophile-lipophile balance value of Tween 20. Moreover, glucose (or single syrup, honey) can be used as a protective agent to mitigate the corrosion of liquefied phenol, and as a thickener to prevent the mold preparation from flowing back out. The molding reagents were quickly injected into the uterus of laying hens by chicken vas deferens, which can prevent fluid outflow and avoid unnecessary mechanical damage caused by other tools. In addition, the laying hens that have been injected with the molding reagent should remain in an inverted state so that the molding reagent can infiltrate the entire fallopian tube.

In the present study, within 48 h after treatments, no hens died in control and LPS group while 2 hens died in LPS + OCR group. There was no difference between LPS group and control group in the appearance of laying hens and eggs. Compared with the LPS group, the LPS + OCR group included 0.5 mL of OCR. So the LPS + OCR group was designed to try to solve problems that the long duration of inflammation, mild symptoms, and lack of susceptibility in the LPS group. The laying hens in LPS + OCR group had some convulsions, pain and restlessness after treatments, and the eggs laid had more blood on the eggshell. The cloaca of laying hens in LPS + OCR group was congested and swollen with the feathers nearby polluted by yellow and white secretions, and the rate of egg production extremely decreased. This result may be because the main component, phenol, a chemical reagent, is highly corrosive to skin and mucous membrane, etc. After injection into the fallopian tube, it can cause damage to the mucous membrane of fallopian tube and secondary infection of various pathogenic microorganisms to cause salpingitis, which is similar to the inflammation of naturally occurring salpingitis in laying hens.

Pathological observation of the fallopian tube in laying hens after treatments showed that the secondary villus length in the magnum of oviduct in LPS + OCR group was shorter, and the primary villi area was reduced due to the reduction of the width of primary villi. Second, the isthmus villus length was also reduced in LPS + OCR group. Finally, the intervillous space of uterus was enlarged, indicating that the villi density in the uterus was reduced. The morphological damage of villus in the oviduct of laying hens in LPS group was lighter than that in LPS+OCR group. This may be related to the fallopian tube has a certain tolerance to *Escherichia coli*, so the effects of LPS treatment were not ideal.

In order to further determine the effectiveness of simulating salpingitis method, the expression of inflammatory-related factors in the oviduct was detected. IL-6 is a multifunctional cytokine that primarily mediates the acute phase response, an innate immune mechanism triggered by infection and inflammation (Akira et al., 1990). IFN- $\gamma$  can enhance the activity of T lymphocytes, macrophages and natural killer cells to achieve a better immune response. Mammalian TNF- $\alpha$  is a member of a family of NF- $\kappa$ B activated signaling cytokines that stimulate systemic inflammation (Lu et al., 2009). iNOS, mainly produced by macrophages, catalyzes the production of NO, which plays a key role in the immune system as a cytotoxic and tumoricide (Lin et al., 1996). In the current study, LPS + OCR group showed the highest mRNA expression of inflammatory factors, including IL-6 and IFN- $\gamma$  in magnum, IFN- $\gamma$  in isthmus, IFN- $\gamma$ , TNF- $\alpha$ , and iNOS in the uterus, respectively.

In conclusion, the results of morphological damage of fallopian tube tissue and increased expression of inflammatory factors in LPS+OCR group suggested that LPS + OCR treatment can provide data basis to establish salpingitis model in laying hens for studying the pathogenesis of it.

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## DISCLOSURES

The authors declare that there is no conflict of interest.

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