Research Note: Disturbance of intracellular calcium signal in salpingitis simulation of laying hens

L. L. Li ,[†] Z. P. Liu,[†] C. A. Liu,[†] S. S. Elnesr ,[‡] S. S. Guo,[†] B. Y. Ding,[†] and X. T. Zou*,[1]

*Key Laboratory of Molecular Animal Nutrition (Zhejiang University), Ministry of Education, China; [†]Hubei Key Laboratory of Animal Nutrition and Feed Science, Wuhan Polytechnic University, Wuhan 430023, China; and [‡]Department of Poultry Production, Faculty of Agriculture, Fayoum University, Fayoum 63514, Egypt

ABSTRACT This study investigated whether there is disturbance of calcium signal in the simulated salpingitis of laying hens. A total of 90 Roman Pink layers (81 wk; 1.916 ± 0.17 kg) were divided into 3 groups (Control treated with PBS, 1.85 mg lipopolysaccharide (**LPS**)/layer as LPS group, 1.85 mg LPS/layer as LPS +organic chemical reagent (**OCR**) group) with 6 replicates of 5 layers. Compared with the Control, the mRNA expression of calcium/calmodulin dependent protein kinase IV (**CaMK IV**), sarcoplasmic/endoplasmic reticulum calcium ATPase (**SERCA**), and plasma membrane calcium-transporting ATPase (**PMCA**) were not only decreased ($P < 0.05$) in magnum of laying hens from LPS and LPS+OCR groups, but also in isthmus and uterus of hens from LPS+OCR group. Moreover, the mRNA expression of calcium sensing receptor (**CaSR**) and Orai1 in uterus from LPS+OCR group were higher ($P < 0.05$) than that from Control. The relative fluorescence intensity of Ca^{2+} in uterus from LPS and LPS+OCR groups were significantly higher than that from Control ($P < 0.05$). In conclusion, it existed that the linkage of simulated salpingitis treated with LPS+OCR and altered intracellular calcium signals in layers, which provided a new insight for alleviating salpingitis and uterine dysfunction of laying hens.

Key words: calcium signal, salpingitis, uterus, lipopolysaccharide, laying hen

2023 Poultry Science 102:102226
https://doi.org/10.1016/j.psj.2022.102226

INTRODUCTION

Salpingitis, a common disease of laying hens in large-scale breeding mode, is mainly caused by external Gram-negative bacteria such as *Escherichia coli* and *Salmonella* invading the fallopian tubes through cloaca. Lipopolysaccharide (**LPS**), a major component of the outer membrane of gram-negative bacteria, can possess powerful biological functions and as an efficient stimulator in the immune system.

As a part of the fallopian tube, the uterus is the site of eggshell formation. In the process of infection from the bottom to the top of the reproductive tract, uterus is more vulnerable to be damaged, resulting in uterine edema, bleeding, and serious decline in eggshell quality. At present, the mechanism of the decline in eggshell quality caused by salpingitis is still unknown.

Ca^{2+} is an important intracellular second messenger, which plays an important role in regulating various cellular processes such as cell contraction, secretion, gene transcription, cell growth, cell differentiation, and death (Raffaello et al., 2016). Intracellular Ca^{2+} ($\mathbf{[Ca^{2+}]_i}$) mainly comes from extracellular Ca^{2+} influx and release of Ca^{2+} stored in the endoplasmic reticulum (**ER**). In the inositol 1,4,5-trisphosphate ($\mathbf{IP_3}$) -Ca^{2+} signaling pathway, IP_3 mediates Ca^{2+} release in ER by binding to IP_3 receptor ($\mathbf{IP_3R}$) on ER membrane, leading to depletion of $[Ca^{2+}]_i$ storage and decrease of Ca^{2+} level in ER (Berridge, 2016). Storage operated cation channels activated by Ca^{2+} release is then initiated and Ca^{2+} is entered from the extracellular space through storage operated cation channels (Kim et al., 2013), such as transient receptor potential channel 1 (**TRPC1**). Stim1 senses low calcium concentration in the ER and activates Orai1 to increase $[Ca^{2+}]_i$ and replenish the calcium storage in ER (Dalal et al., 2020). In addition, it is inferred that the p38-MAPK pathway may play a physiological role by changing cellular calcium ion concentration (Han and Lee, 2005).

Therefore, the objective of this study was to explore whether there is disturbance of calcium signal in the process of eggshell quality decline caused by salpingitis of

Received July 30, 2022.
Accepted September 29, 2022.

[1]Corresponding author: xtzou@zju.edu.cn

laying hens by investigating the effects of salpingitis simulation of laying hens on expression of genes associated with calcium signaling in oviduct of laying hens.

MATERIALS AND METHODS

Materials

LPS: Sigma, SL263003. Organic chemical reagent (**OCR**): 25% liquefied phenol (10015318) + 2.5% Tween 20 (30189328) + 2.5% Span 20 (30170428) + 10% glucose (63005518) + 60% PBS.

Birds, Diets and Management

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).

Ninety 81-wk-old Roman Pink laying hens (average weight: 1.916 ± 0.17 kg;average egg production rate: 76%) with good physical condition (without the oviduct disease) were randomly allocated into 3 groups, comprising control (treated with PBS), LPS group (1.85 mg/mL) and LPS+OCR group (3.7 mg/mL; LPS:OCR (v:v) = 1:1). Each of groups consisted of 6 replicates (5 birds/replicate, 1 bird/cage). The size of each cage (equipped with 2 nipple drinkers and 1 feeder) was 45 × 45 × 45 cm^3. All hens were housed in an enclosed, ventilated, and conventional house with 16 h lighting. A basal diet shown in Table 1 was fed during 2-wk adaption period and all experimental period. Feed and water were offered ad libitum.

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

Ingredients (%)	Content	Calculated nutrient levels (%)	Content
Corn (7.8% CP)	20.00	ME (kcal/kg)	2,713
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[1]	0.02		
Trace mineral premix[2]	0.10		
Compound enzyme preparations	0.02		
Total	100		

[1]Provided per kilogram of diets: vitamin A 10,000 IU, vitamin D_3 2,500 IU, vitamin E 26 mg, vitamin B_1 2.0 mg, vitamin B_2 6.0 mg, vitamin B_6 3.0 mg, vitamin B_{12} 0.025 mg, D-Biotin 0.050 mg, folic acid 1.0 mg, pantothenic acid 10 mg, niacin acid 30 mg.

[2]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.

Simulation of Salpingitis in Laying Hens

The experimental method was consistent with that reported by Fang et al. (2021). First, laying hens with its feet and wings fixed were kept upside down; then, press the abdomen near the cloaca to make ectropion and exposure of the uterus apertura, which the prepared reagents for simulating salpingitis were poured into with the chicken vas deferens (1 mL/layer); finally, the injected chickens were kept in an inverted position for 5 to 10 min to allow the reagents to fully upstream flow the entire fallopian tube wall.

Sample Collections

After 48 h of treatment, one bird from each group randomly selected was fasted overnight for up to 12 h and sacrificed humanely by CO_2 asphyxiation to collect samples. Part of uterus (1 × 1 cm) isolated was fixed in 4% paraformaldehyde and then treated with ethanol and xylene, embedded in paraffin for the assessment of Ca^{2+} signals. Another part of uterus samples (1 × 1 cm) were frozen immediately in liquid N_2 and stored at −80°C for further analysis.

Quantitative Real-Time Polymerase Chain Reaction

The relative transcript levels were measured for genes associated with calcium signaling. Primers used for real-time quantitative fluorescence PCR analysis were followed from 5′→3′: Ca^{2+}/calmodulin (**CaM**)-dependent protein kinases (**CaMKs**): F: ATGTGCCTATGGCCCTGAAG, R: TTTGCCGCTTTTCCTGTGAC (NM_001034813.1); calcium-sensitive receptor (**CaSR**): F: TGGAACACTGCTGCCTATGG, R: ATGCACTCCACTGATTCGGG (XM_040661543.1); Orai1: F: CTCTGCCTCCGGAAAGAGCTG, R: ATGCTCGTTCAGGCTCATGG (NM_001030658.2); IP_3R: F: GTCCTGAATCCTGTGAACGC, R: ACCACCTCTACCTCCCACAG (NM_001174059.1); SERCA: F: GGCCCGTAACTACCTGGAAC, R: CCAGATAACCAAGGGCAGGG (NM_001271974.1); PMCA: F: TCACAGTCATCAGAGGTGGC, R: GCTGCACCATCTTGAGCTTT (NM_001168002.3); calreticulin (**CRT**): F: GTGCTCATCAACAAGGACATC, R: CCATTCCCCATCCATCTC (XM_040693083.1); p38: F: GCCAAAAGGACCTACCG, R: GAGCCAAGCCAAAATCC (XM_040691291.1); TRPC1: F: TCTCAAAGTAGTTGCCCATAA, R: AAATACCCGCACAGTCCC (XM_040705527.1) and β-actin: F: GAGAAATTGTGCGTGACATCA, R: CCTGAACCTCTCATTGCCA (NM_205518). Total RNA was extracted from the uterus using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The quality and quantity of RNA were assessed using a NanoDrop, ND-2000 UV-VIS spectrophotometer (Thermo Scientific, Wilmington, DE) at 260 and 280 nm. The cDNA synthesis used 1 μg of RNA with the PrimeScript RT reagent kit (Takara Biotechnology (Dalian) Co., Ltd.,

Dalian, China) according to the manufacturer's instructions. There were 6 samples for each group, and each sample was performed in triplicate. Real-time PCR (Applied Biosystems 7500 Real-time PCR System; Applied Biosystems, Foster, CA) was performed according to the following protocol: 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. PCR of β-actin was used to normalize and quantitate the mRNA levels of the target genes using the comparative CT method ($2^{-\Delta\Delta CT}$).

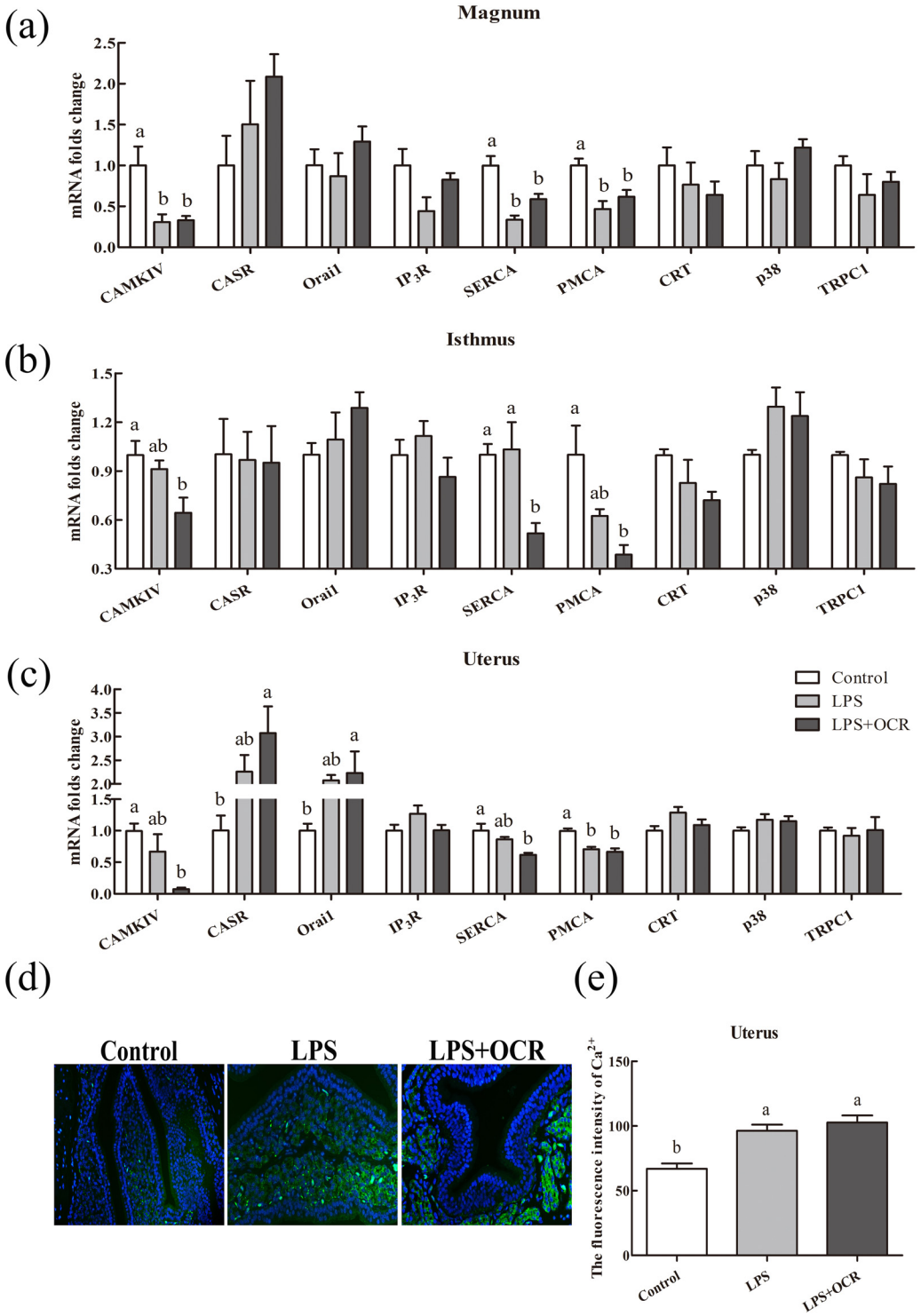

Figure 1. Expressions of calcium signal-related genes mRNA in magnum (a), isthmus (b), and uterus (c) quantified with real-time PCR and the fluorescence intensity of Ca^{2+} in the uterus of oviduct of laying hens (d, e). The Ca^{2+} signal in the uterus was stained with Fluo-4 AM and determined by laser scanning confocal microscope. β-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 ($P < 0.05$). Abbreviations: CaMK IV, calcium/calmodulin dependent protein kinase IV; CRT, calreticulin; CaSR, calcium sensing receptor; IP_3R, inositol 1,4,5-trisphosphate receptor; LPS, lipopolysaccharide; OCR, organic chemical reagent; PMCA, plasma membrane calcium-transporting ATPase; SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase; TRPC1, transient receptor potential channel 1.

Ca^{2+} Signals Measurement

Ca^{2+} signals in uterus were measured with Fluo-4 AM (Beyotime, Shanghai, China) according to manufacturers' instructions. In detail, paraffin section of uterus were incubated with Fluo-4 AM (final concentration of 1 μM) for 30 min in PBS at 37°C, then washed three times with PBS and incubated for an additional 15 min in the absence of Fluo-4 AM to complete the de-esterification process of the dye. The fluorescent intensity was obtained by laser scanning confocal microscope and Image J.

Statistical Analyses

All experimental data were presented as means with SEM and analyzed statistically by one-way ANOVA using SPSS 20.0. A Tukey 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 AND DISCUSSION

In our previous study (under review, unpublished), it was found that LPS+OCR treatment had morphological damage of oviduct and increased expression of inflammatory factors in magnum, isthmus, and uterus, which indicated that LPS+OCR treatment can provide data basis to establish salpingitis model in laying hens for studying the pathogenesis of it.

Since SEACR controls intracellular Ca^{2+} influx into the ER and PMCA controls intracellular Ca^{2+} outflow into the extracellular matrix (Berridge et al., 2003), which are secreted into the uterine cavity after binding with bicarbonate ions for eggshell calcification (Zhang et al., 2015). The results in current study that laying hens in LPS+OCR group had downregulated mRNA levels of SERCA and PMCA in magnum, isthmus and uterus compared to those in Control ($P < 0.05$; Figure 1A−1C suggest that increased intracellular Ca^{2+} concentration exists in LPS+OCR simulated salpingitis of laying hens.

So many downstream signaling pathways activated by $[Ca^{2+}]_i$ changes regulate numerous cellular processes by binding to calcium-binding proteins, effector proteins, and transcription factors (Berridge et al., 2003). Changes in Ca^{2+} concentration first cause changes in the conformation and activity of CaM, and then activate target proteins containing CaM binding sites, such as CaMK IV (Stratton et al., 2013). CRT is a Ca^{2+} binding protein in ER lumen, which has a variety of functions, involving in calcium homeostasis, cell adhesion, protein folding, and other cellular signaling pathways (Wang et al., 2012). CaSR is a type C G-protein-coupled receptor that can detect extracellular calcium ion levels (Huang et al., 2016).

In present study, laying hens in LPS+OCR group had upregulated mRNA levels of CaSR and Orai1 in uterus and downregulated mRNA levels of CaMK IV in magnum, isthmus, and uterus compared to those in Control ($P < 0.05$; Figure 1A−1C, which suggests that CaMK IV, CaSR, and Orai1 might be involved in disturbance of calcium signal in LPS+OCR-simulated salpinitis of laying hens. The result was not in according with Cuschieri et al. (2005) who reported that regardless of whether pretreated with platelet activating factor, inhibition of CaMK IV inhibits LPS-induced activation of ERK 1/2, JNK/SAPK, NF-KB, and AP-1 and TNF-α production. However, the mechanism of calcium-binding protein involved in the increase of $[Ca^{2+}]_i$ is still unclear, especially the mechanism of CaMKs-Ca^{2+} signaling pathway mediating the effects of salpingitis on eggshell quality of laying hens needs further study.

Since the uterus is the site of eggshell formation affected by salpingitis, to verify the above results of increased $[Ca^{2+}]_i$ in laying hens simulated salpingitis, we analyzed the fluorescence intensity of Ca^{2+} in the uterus of oviduct of laying hens using Ca^{2+} fluorescence probe. The results of this experiment show that laying hens in LPS+OCR group had higher fluorescence intensity of Ca^{2+} in the uterus than that in Control ($P < 0.05$; Figures 1D and 1E, which was corresponding to results of expressions of genes related to calcium signal in uterus. Moreover, the better effects on expressions of genes related to calcium signal in magnum, isthmus, and uterus were found in LPS+OCR group compared with LPS group, which indicated that LPS+OCR treatment had greater simulation effects than single LPS treatment.

In conclusion, it existed that the linkage of simulated salpingitis treated with LPS+OCR and altered intracellular calcium signals in layers. Moreover, the effect of calcium signal-related gene expression disturbance in simulated salpingitis was better in LPS+OCR group than in LPS group. The current study provided a new insight for alleviating salpingitis and uterine dysfunction of laying hens.

ACKNOWLEDGMENTS

This work was supported by Key Laboratory of Molecular Animal Nutrition of Ministry of Education (KLMAN202202) and the Hubei Provincial Key R&D Project (2022BBA0014) .

DISCLOSURES

The authors declare that there is no conflict of interest.

REFERENCES

Berridge, M. J., M. D. Bootman, and H. L. Roderick. 2003. Calcium signalling: dynamics, homeostasis and remodelling. Nat. Rev. Mol. Cell Biol. 4:517–529.

Berridge, M. J. 2016. The inositol trisphosphate/calcium signaling pathway in health and disease. Physiol. Rev. 96:1261–1296.

Cuschieri, J., E. Bulger, I. Garcia, S. Jelacic, and R. V. Maier. 2005. Calcium/calmodulin-dependent kinase II is required for platelet-activating factor priming. Shock. 23:99–106.

Dalal, P. J., W. A. Muller, and D. P. Sullivan. 2020. Endothelial cell calcium signaling during barrier function and inflammation. Am. J. Pathol. 190:535–542.

Fang, H., H. Quan, Y. Zhang, Q. Li, Y. Wang, S. Yuan, S. Huang, and C. He. 2021. Co-Infection of *Escherichia coli, Enterococcus faecalis* and *Chlamydia psittaci* contributes to salpingitis of laying layers and breeder ducks. Pathog 10:755.

Han, H. J., and Y. J. Lee. 2005. Insulin stimulates Ca^{2+} uptake via PKC, cAMP, and p38 MAPK in mouse embryonic stem cells. Life Sci. 76:2903–2919.

Huang, B., D. Xiao, B. Tan, H. Xiao, J. Wang, J. Yin, J. Duan, R. Huang, C. Yang, and Y. Yin. 2016. Chitosan oligosaccharide reduces intestinal inflammation that involves calcium-sensing receptor (CaSR) activation in lipopolysaccharide (LPS)-challenged piglets. J. Agric. Food Chem. 64:245–252.

Kim, M. H., J. B. Seo, L. A. Burnett, B. Hille, and D. Koh. 2013. Characterization of store-operated Ca^{2+} channels in pancreatic duct epithelia. Cell Calcium. 54:266–275.

Raffaello, A., C. Mammucari, G. Gherardi, and R. Rizzuto. 2016. Calcium at the center of cell signaling: interplay between endoplasmic reticulum, mitochondria, and lysosomes. Trends Biochem. Sci. 41:1035–1049.

Stratton, M. M. L. H., H. Schulman Chao, and J. Kuriyan. 2013. Structural studies on the regulation of Ca^{2+}/calmodulin dependent protein kinase II. Curr. Opin. Struct. Biol. 23:292–301.

Wang, W. A., J. Groenendyk, and M. Michalak. 2012. Calreticulin signaling in health and disease. Int. J. Biochem. Cell Biol. 44:842–846.

Zhang, Q., F. Zhu, L. Liu, C. W. Zheng, D. H. Wang, Z. C. Hou, and Z. H. Ning. 2015. Integrating transcriptome and genome resequencing data to identify key genes and mutations affecting chicken eggshell qualities. PLoS One. 10:e0125890.