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NLRC5 EXPRESSION PROFILE IN THE OVIDUCT OF LAYING HENS AND ITS CHANGES FOLLOWING ESTRADIOL TREATMENT IN INDUCED MOLTING HENS

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ABSTRACT: The innate immune defense system is initiated by recognizing components of microorganisms by different families of receptors such as TLRs and the intracellular NLR family. Among the NLR family, NLRC5 recognizes pathogenic components of microorganisms. The current study aimed to determine the expression profile of *NLRC5* in the mucosal tissue of the oviduct of laying hens and to examine the effects of oviduct regression and estradiol benzoate (EB) on its expression. In this study, we used two groups of laying and molting hens. The molting group was treated by receiving a single dose of sesame oil or estradiol benzoate through intramuscular injection. To examine the histological differences in the oviduct between laying, molting, and estradiol-injected groups, Tissue samples from all segments of the oviduct of all groups were fixed in formalin and stained with hematoxylin and eosin. To observe the profile of *NLRC5* gene and the effects of estradiol treatment, RNA was obtained from the surface epithelium and lamina propria of the whole segments of the oviduct of normal laying hens, and mucosal tissue of magnum, isthmus, uterus, and the vagina of molting groups. Both mucosal surface epithelium and lamina propria expressed *NLRC5*. The expression of *NLRC5* was higher in the molting than in the laying group, and it was lower in the EB group. These results suggested that NLRC5 may have a role in recognizing pathogens invading the oviduct of laying and molting hens, and its expression is changed in association with oviduct growth.

Key words: Chicken oviduct, Molting, Immunity, *NLRC5*, Estradiol benzoate.

INTRODUCTION

Pathogenic microbes can infect the oviduct of laying hens, leading to its dysfunction and the production of contaminated eggs. Many of these microorganisms colonize the cloacal region and may spread to the various oviduct tissues through the vagina (Miyamoto *et al.*, 1997; Reiber *et al.*, 1995).

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The oviduct's innate immune functions serve as the first line of defense against invading microorganisms. (Mageed *et al.*, 2008; Sonoda *et al.*, 2013a). The host defense by innate immunity starts by recognizing specific components of infectious pathogens by specific receptors, then an adequate immune response by synthesizing antimicrobial compounds and/or cytokines is activated to boost the inflammation and to attack the infectious pathogens. We have previously identified the expressions of proinflammatory cytokines (Nii *et al.*, 2011), avian β-defensins (AvBDs) (Mageed *et al.*, 2008), and TLRs (Ozoe *et al.*, 2009) in hen oviduct. Stimulating the tissues of the hen vagina with TLR-specific ligands such as lipopolysaccharide (LPS) or CPG-ODN increased the expressions of interleukin 1B (IL1b), IL6, and AvBDs (Mageed *et al.*, 2008; Mageed *et al.*, 2011; Sonoda *et al.*, 2013b).

Toll-like receptors (TLRs), intracellular nucleotide-binding oligomerization (NOD)-like receptors (NLRs), and retinoid acid-inducible gene-like receptors (RLRs) are the three categories of PAMP recognition receptors that have been identified (Akira *et al.*, 2006; Almeida-da-Silva *et al.*, 2023; Honda and Taniguchi, 2006; Inohara *et al.*, 2005; Kawai and Akira, 2011; Medzhitov, 2007; Saïd-Sadier and Ojcius, 2012). NLRs have three domains: the central nucleotide-binding domain (NOD), the C-terminal leucin-rich repeat (LRP), and the variable N-terminal interaction domain (Marchal *et al.*, 2022). Based on the N-terminal interaction domains, the NLRs are divided into three groups: NLRB with an acidic trans-activating domain, NLRP with a pyrin domain, and NLRC with a caspase recruitment domain (CARD) (Chelbi *et al.*, 2017; Franchi *et al.*, 2009). NLRC5 belongs to the NLRC subfamily, which also includes NLRC1 and NLRC2 (Hughes, 2006; Motta *et al.*, 2015; Proell *et al.*, 2008). It has recently been observed that NLRC5 plays contradictory and contentious roles in regulating the innate and adaptive immune systems in birds (Qiu *et al.*, 2016).Its role and function depends on the type of the cell and the species (Davis *et al.*, 2011) ; NLRC5 positively regulated the IFN pathway (Kuenzel *et al.*, 2010; Neerincx *et al.*, 2010; Wang *et al.*, 2019; Wu *et al.*, 2017; Zhang *et al.*, 2021) and MHC-I (Meissner *et al.*, 2010) in Hela and THP-1 cells, whereas it downregulated the expressions of both IFN and NFkB as well as AP-1 pathway in human embryonic kidney cells 293 (HEK) (Benko *et al.*, 2010). It was reported that the LPS-dependent induction of INFA and INFB in chicken macrophages was mediated by NLRC5 (Lian *et al.*, 2012b). It has been noted that NLRC5 plays a role in MHC-I gene expression, however its effects as an enhancer or suppressor remain unclear (Benkő *et al.*, 2017a, b; Chang *et al.*, 2015; Lupfer *et al.*, 2017; Meissner *et al.*, 2010). Up to date, little information, and publications on the NLRs expression in the peripheral tissues and the oviduct in chickens are available.

In chickens, molting is induced by feed-regulation and various stress, and the oviduct regresses in association with the decline of circulating estrogen level (Nii, 2022). The regressed oviduct may be more susceptible to infection with pathogenic microorganisms, and Salmonella-contaminated eggs are more frequently observed in post-molting hens (Golden *et al.*, 2008). However, it has not been established whether the innate immune mechanisms of the oviduct are also altered in such critical period of the birds. The aim of the present study was to determine the expression profile of *NLRC5* in the oviduct mucosal tissue of laying hens and investigate the impact of oviductal regression and estradiol on its expression.

MATERIAL AND METHODS

Experimental design: In experiment 1, the expression of *NLRC5* was localized in all oviduct segments of laying group by RT-PCR in separated samples of the surface epithelium and lamina propria. In experiment 2, effects of oviduct regression on the *NLRC5* expression were examined by comparison between laying and molting hens, and effects of estradiol were examined using estradiol- or vehicle-treated molting hens by quantitative PCR.

Experimental birds: In the current study, 500-day-old white leghorn hens that were laying four eggs or more in one clutch were used. They were housed in separate cages with light for 14 hours and darkness for 10 hours. They were fed *ad libitum* with free access to water (laying group). To induce molting, we use the same method of our previous study (Ariyadi *et al.*, 2013). Some of the hens were subjected to feeding restriction by providing them with 25 g/day of feed and free access to water. The molting hens were used on the day 20 of egg laying cessation when the oviduct was completely regressed (molting group).

To investigate the impact of estradiol on NLRC5 expression, hens in the molting group were given an intramuscular injection of 1 mg/kg BW of estradiol-benzoate or 100 µl/kg BW of sesame oil 24 hours before use (EB and oil-control groups). B-estradiol-benzoate (Sigma-Aldrich Co., St. Louis, MO, USA) was dissolved in sesame oil to prepare a stock solution with a 10 mg/ml concentration. The Hiroshima University Animal Research Committee (No. C11-4) approved this study, and the handling of the birds was conducted in compliance with committee regulations.

Histological differences of the oviduct between laying, molting and estradiol-injected groups: The oviducts of all groups were removed (n= 3) and washed with buffered-saline solution. Then it was perfused and soaked in formalin (10% (vol/vol)) for overnight. Small parts of the middle region of each oviduct segments (from the infundibulum to the vagina) were collected, trimmed, kept in perforated cages and kept in formalin. After fixation was performed, they were

soaked in ascending grades of ethanol for dehydration, then the ethanol was replaced by xylene and then embedded in melted paraffin to prepare blocks. Section of 4 µm-thick were prepared on slides and air-dried, deparaffinized in xylene, rehydrated, and stained with hematoxylin before being washed in tap water and stained with eosin, dehydrated ethanol, cleared in xylene, finally mounted by Canada balsam and cover glass.

Identification of NLRC5 expression profile in the mucosal epithelium and lamina propria of the oviduct: The mucosal tissues of each segment of the oviduct were collected from laying hens (n = 3) and embedded in cryo-embedding OCT compound (SAKURA Fine Technical Co. Ltd., Tokyo, Japan) and then frozen in a mixture of isopentane and solid CO2. Frozen sections of thickness 8 µm were prepared using cryostat (Bright Instrument Co. Ltd.), dried, then they were fixed in acetone at a temperature of -20°C for 30 sec. They were then washed in ultrapure water (30 sec x 2), dehydrated in ascending grades of ethanol, and were embedded in xylene for 5 min at room temperature for drying. Cells from mucosal epithelial surface and lamina propria tissues of different segments were collected separately by using laser microdissection using a laser capturing system ARCTURUS LM200 PXL-220 PIXCELL II (Arcturus Bioscience, Inc., Mountain View, CA, USA). Briefly, a macro-LCM caps (Capsure, Arcturus Bioscience Inc., CA., USA) were placed on the sections, and a 15-µm-diameter laser beam was applied to the mucosal surface epithelium or lamina propria to capture them on the caps. Approximately 200 laser spots were used to obtain cells from both the lamina propria and epithelial surface. Total RNA was extracted, and genomic DNA was removed by using RNAGEM kit (Zygem Co. Ltd. Hamilton. New Zealand using thermal cycler (MJ Research Inc., Waltham, MA., USA) in accordance with the manufacturer's instructions. Reverse transcription for RNA was done with ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan), the mixture of the reaction composed of 10 µl contained 5.5 µl of total RNA, 0.5 µg of oligo (dT) 20 primer, 1x RT buffer, 1 mM dNTP mixture, 20 U RNase inhibitor and 50 U ReverTra Ace. Then reverse transcription was done in PTC-100 Programmable Thermal Controller (MJ Research Inc.) the conditions of the reverse transcription were adjusted as follow: 42 °C for 30 min followed by 99°Cfor 5 min. The PCR amplification was performed using Takara Ex Taq (Takara Bio. Inc., Shiga, Japan) and PTC-100 Programmable Thermal Controller (MJ Research Inc.) using the primers shown in Table 1. A volume of 25 µl of the PCR reaction mixture included 0.5 µM of each primer, 0.5 µl of cDNA, 1x PCR buffer, 0.2 mM of dNTP, 1.25 U Takara Ex Taq. The conditions of the PCR amplification were an initial step for 30 sec at 94°C. Then 50 cycles each composed of denaturation step for 30 sec at 95°C, annealing step for 30 sec at 65°C, and extension step for 1 min at 72°C. Then, the products of the PCR were

subjected to electrophoresis in 2% (w/v) of the agarose gel mixed with ethidium bromide solution 0.025% (w/v).

Table 1. Primers sequences of *NLRC5* and *RPS17*

Target gene	Sequence 5′–3′	Accession number	Reference
NLRC5	F: TGAGCTACACGTCAGGAAGGA R: GCTCTGCAGAATGGACACAA	JQ044414	(Lian *et al.*, 2012a)
RPS17	F: AAGCTGCAGGAGGAGGAGAGG R: GGTTGGACAGGCTGCCGAAGT	NM_204217	(Abdel-Mageed *et al.*, 2016)

Abbreviations: F, forward; R, reverse.

Real-time PCR analysis of NLRC5 expression in the segments of the oviduct of laying, molting and estradiol benzoate-injected molting hens: In experiment 3, the mucosal tissue of the magnum, isthmus, uterus and the vagina of the laying, molting, EB and oil-control groups were collected (n= 5 each). Total RNA from those segments was extracted using Sepasol-RNA I Super (Nacalai Tesque Inc., Kyoto, Japan), then the RNA was dissolved and kept in Tris-EDTA (TE) buffer composed of 10 mM Tris and 1 mM EDTA with pH 8.0. The extracted RNA samples were mixed with 1 U of RQ1 RNase-free DNase (Promega Co., Madison, WI, USA), and put into the programmable thermal controller mentioned above for 45 min at 37°C and 10 min for 65°C. The concentration of RNA extracted from all samples was measured with Gene Quant Pro (Amersham Pharmacia Biotech, Cambridge, UK). To examine the change in the mRNA of *NLRC5*, real-time PCR for all samples of the experimental groups was done by a Roche Light Cycler Nano system (Roche Applied Science, Indianapolis, IN, USA) using a volume of 20 µl of the PCR mixture containing 3 µl of the cDNA, 1×Thunderbird SYBR qPCR mix (Toyobo), 1×ROX reference dye, and 0.5 µM of both forward and reverse primers. The mixture was moved into 20 µl capillaries (Roche Diagnostics GmbH., Mannheim, Germany). The cycle parameters used for the PCR were 95 °C for 5 sec and 62 °C for 20 sec. The data obtained from the real-time PCR were analyzed by the $2^{-\Delta\Delta CT}$ CT method to calculate the relative level of mRNA in each sample using *RPS17* as the housekeeping gene (Livak and Schmittgen, 2001). For real-time standardization, samples of each segment of the oviduct of the laying groups were used as a standard for the analysis of differences in the expressions between laying and molting groups, whereas sample from an oil-control group was used as the standard for the comparison between EB and oil-control groups.

The results were shown as the relative expressions obtained from the ratio between the samples of the experimental groups and the standard sample. The PCR products of *NLRC5* in each sample were also electrophoresed as described

in Experiment 1. For statistical analysis, SPSS 21 was used. Relative expression of *NLRC5* was expressed as the mean ± standard error of mean (M ± SEM). Student t test was considered to and examine the significant differences between the laying and molting groups, and between the oil-control and EB groups. Statistical difference was established at $P< 0.05$.

RESULTS AND DISCUSSION

Fig.1 illustrates the histological differences of the oviduct among the three groups of the experiments, namely, the laying, molting and EB groups. In molting birds, the mucosal epithelial surface in all oviduct segments was shorter than the laying hens and EB-treated birds. Also, the lamina propria in the magnum, isthmus and uterus of molting hens showed a dramatic regression especially in the tubular glands with clearly identified blood capillaries as well as more leukocyte aggregations in all segments of molting hens.

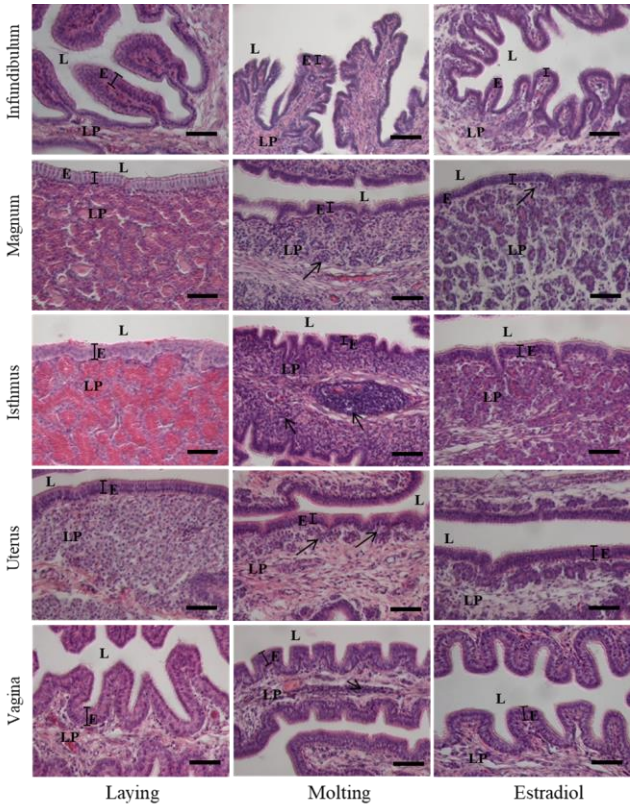

Fig. 1. Sections of all oviduct segments of laying, molting, and estradiol benzoate-injected hens (n= 3) were stained with hematoxylin and eosin (HE). The height of the mucosal surface epithelium is lower in molting and estradiol benzoate-injected hens (EB) than in laying hens. Arrows indicate leukocyte aggregations. The pattern of the tubular glands in the molting group's lamina propria (LP) is more regressed than in the EB group and non-treated group. L, lumen; E, mucosal surface epithelium; Scale bar= 50 μm.

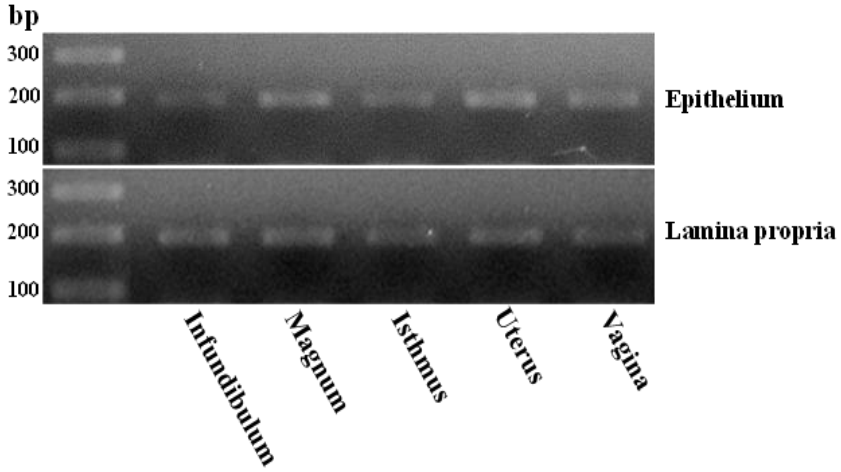

Fig. 2. Expression profile of NLRC5 in the surface epithelium and lamina propria of mucosal tissue in oviduct segments of laying hens. The specific PCR products were electrophoresed and separated on 2% agarose gel.

The specific bands of the PCR products of *NLRC5* were observed in both the mucosal epithelium and lamina propria of all oviduct segments from the infundibulum to vagina of laying hens (Fig. 2).

To our knowledge, the present observation is the first to declare that *NLRC5*, that is a member of NLR family, is clearly expressed in the hen oviduct. In the present work, *NLRC5* expression was seen in the oviduct mucosa in both the surface epithelium and lamina propria. The cells of the surface epithelial tissues in the oviduct composed of two different types, namely, the elongated apical and the short basal cells, whereas the lamina propria layer consists of connective tissues containing fibroblasts and blood capillaries. Glandular cells are also developed in the magnum, isthmus, and uterus.

Previous studies identified T and B lymphocytes, and macrophages immunocompetent cells in both surface epithelium and lamina propria of the oviduct (Das *et al.*, 2005; Zheng and Yoshimura, 1999; Zheng *et al.*, 1998).

Although this study did not identify specific cells that express *NLRC5* within the oviduct, it is possible that some of the cells that make up the mucosal epithelium and lamina propria express it.

Microorganisms such as *Salmonella sp* were observed colonizing the cloaca (Alig *et al.*, 2023), and may ascend the lumen inside the oviduct of the laying hen (De Buck *et al.*, 2004; DeSantis *et al.*, 2006; Jiang *et al.*, 2023; Miyamoto *et al.*, 1997). Such *Salmonella* organisms may not be located in the oviduct lumen only, but also may invade the cytoplasm of surface mucosal epithelium cells of the oviduct (Castanheira and García-Del Portillo, 2017; Takata *et al.*, 2003) and

the cells of the tubular gland (De Buck *et al.*, 2004). *Salmonella* organisms colonizing the digestive tracts may be captured by macrophages and transported to the lamina propria of the oviduct through blood circulation. Since NLRC5 is synthesized and located in the cytoplasm (Wicherska-Pawłowska *et al.*, 2021; Yao and Qian, 2013), it may recognize the intracellular microorganisms that invade the cytoplasm of cells. Although the ligands of NLRC5 have not been fully established in chicken, it interacted with LPS and Polyinosinic-polycytidylic acid (Poly I:C) in chicken macrophages (Lian *et al.*, 2012b). Fig. 3 shows the differences in the levels of *NLRC5* expression in the mucosal tissue of magnum, isthmus, uterus, and vagina between healthy laying hens and molting hens. In the magnum, the *NLRC5* expression was about five-fold higher in the molting than laying group. In the isthmus and uterus, the *NLRC5* expression levels were two-fold greater in molting group than laying group, whereas in the vagina, there were no significant differences in the *NLRC5* expression levels between laying and molting groups. In mammals, it was reported

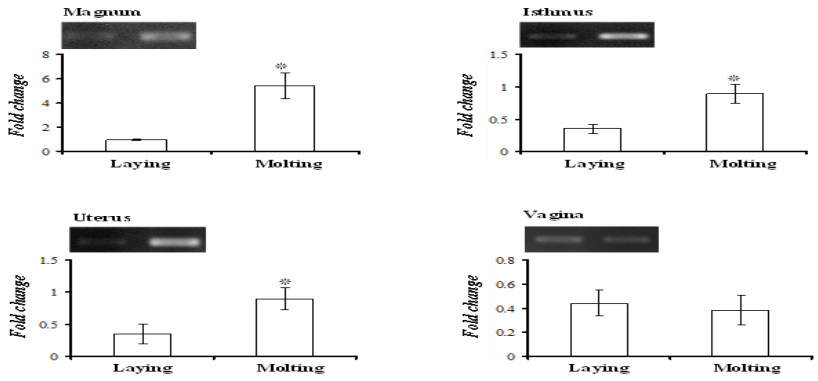

Fig. 3. Expression levels of *NLRC5* in the magnum, isthmus, uterus, and vagina of laying and molting hens. *indicating a significant difference between laying hens and molting hens ($P<0.05$). Values representing the SEM of the fold changes (N = 5).

that NLRC5 recognizes LPS (Wicherska-Pawłowska *et al.*, 2021; Yao and Qian, 2013) in murine cells (Benko *et al.*, 2010), Poly I:C in murine and human cells (Benko *et al.*, 2010; Neerincx *et al.*, 2010; Yao and Qian, 2013) and viral ssRNA and dsRNA in human cells (Fekete *et al.*, 2018; Neerincx *et al.*, 2010). It is thought that NLRC5 expressed in the mucosal surface epithelium and lamina propria cells recognizes patterns of microorganisms invading the oviduct mucosa.

The level of *NLRC5* expression was higher in molting compared with laying hens; during molting, oviduct secretory function is decreased, and gene expression level encoding the secretory substances may be decreased. Thus, we

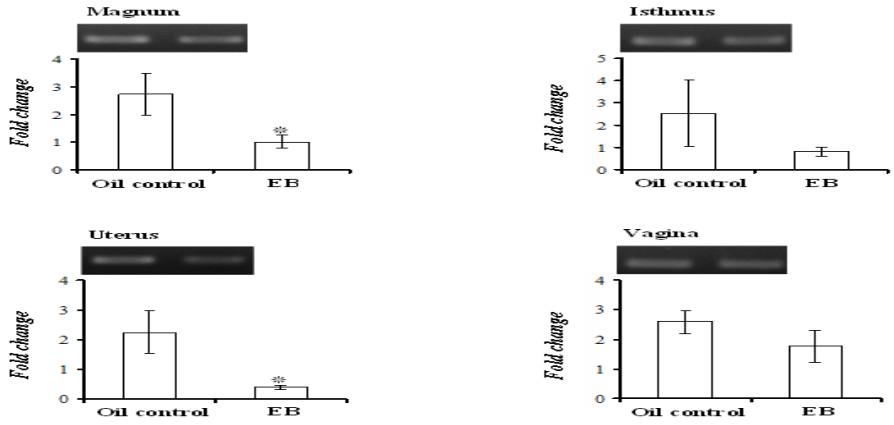

Fig. 4. Expression levels of NLRC5 in the magnum, isthmus, uterus, and vagina of molting hens treated with or without estradiol benzoate (EB). *indicating a significant difference between EB and sesame oil-control groups (P<0.05). For more details, refer to Figure 3.

suppose that relative level of *NLRC5* mRNA in the total mRNA might appear greater in molting than laying. It was reported that the mid region of the stromal tissue in the molting hens showed a higher density of infiltrated immunocompetent cells compared to healthy laying hens (Yoshimura *et al.*, 1997). If those cells express *NLRC5*, they may be the source responsible for the increased expression of *NLRC5* during molting. In the present observations, more aggregations of cellular infiltrations were found in the molting group than laying group (Plate 1). Hence, we hypothesize that those aggregations of cells are the source of the increased *NLRC5* in the molting groups.

Figure 4 demonstrates the levels of *NLRC5* expression in the oviduct from the magnum to the vagina of oil-control and EB groups. The magnum and uterus of the EB group expressed a significantly lower level of *NLRC5* when compared to the oil-control group. On the other hand, the expression levels in the isthmus and vagina showed no significant differences between the two groups.

It was indicated that sex steroid hormones have modulating effects on the immune functions (Barua and Yoshimura, 1999; Bilbo and Nelson, 2001; Foo *et al.*, 2017; Hoffmann *et al.*, 2023; Yoshimura, 2004). Hence, we assume that the immune functions of the oviduct were increased after injecting molting hens with estradiol leading to decrease in the intensity of the microorganisms infecting the oviduct, and then it may cause lowering the expression of *NLRC5*.

LPS upregulated the expression of TLR4 as well as the proinflammatory cytokines and the chemokines (Ciesielska *et al.*, 2021; Nii *et al.*, 2011), and increased the infiltration of T cells in the hen oviduct (Nii *et al.*, 2013). Both the

intracellular TLRs and NLRs families are Pathogen Recognition Receptors (PRRs) that enhance inflammatory response and apoptosis, leading to rapid elimination of the invasive pathogens (Yao and Qian, 2013). In a previous study using chicken macrophage cell line in which *NLRC5* was knocked down, LPS was found to upregulate *INF-a* and *-b* (Lian *et al.*, 2012b). Infection of the oviduct tissues with *Salmonella enteritidis*, an invasive pathogen, is reported more frequent during molting phase (Holt, 2003; Ricke, 2003). Although the change in *TLRs* expression during molting are not known, the expression of *TLR2* and *-4* was increased with respect to sexual maturation (Michailidis *et al.*, 2011). It is assumed that not only TLRs but NLRs including NLRC5 recognize microbes in both the regressed molting hens and healthy laying hens.

We conclude that this study revealed the presence of *NLRC5* expression in the mucosal tissue of the oviduct in healthy laying and molting hens. In the molting group, the levels of *NLRC5* expression were higher compared to the laying group. On the other hand, the expression levels were lower in estradiol benzoate-treated group compared to oil control group. This study suggests that NLRC5 may be involved in recognizing the pathogens that may invade the oviduct tissues of both laying and molting hens and its expression is subjected to change with the growth of the oviduct.

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