Time course effect of lipopolysaccharide on Toll-like receptors expression and steroidogenesis in the Chinese goose ovary

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Abstract

The ovary of Chinese goose is easily infected by microorganisms because of the mating behaviour in water, which causes decreased laying performance. This study investigated the time course effect of lipopolysaccharide (LPS) on the steroidogenesis and mRNA expression of Toll-like receptors (TLRs), a class of key pattern recognition receptor, in the breeding goose ovary. The laying geese were treated intravenously with LPS for 0, 6, 12, 24 and 36 h, and all birds were slaughtered approximately 8 h after oviposition. The expression levels of TLRs in the white and yellowish follicles, and granulosa and theca layers of hierarchical follicles were examined by real-time PCR. All 10 members of avian TLR family were differentially expressed among the different follicular tissues. Moreover, at 24 and 36 h after LPS treatment, the hierarchical follicle morphological structure was altered, but the expression levels of TLRs were still higher than the control. Furthermore, during LPS treatment period, the expression pattern of TLRs 2A and 4 genes was similar to that of TLR15 in the white follicles, TLRs 1B, 5 and 15 in the yellowish follicles, TLRs 7 and 15 in the granulosa layer, and TLRs 1A, 2B, 3, 7 and 15 in the theca layer, which had a negative correlation with the kinetics of plasma P4 and E2 concentrations. In conclusion, the mechanism by which pathogen infection inhibited goose follicular growth and further decreased egg production may involve a gradually enhanced inflammatory response and reduced endocrine function. This may be due to stimulated TLRs in the ovary.

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Introduction

Similar to the domestic chicken ovary, the goose ovary contains numerous prehierarchical and preovulatory follicles that ovulate on successive days (Qin *et al.* 2013); however the female goose could lay only 50–60 eggs throughout the course of the breeding season. In addition, the ovary is susceptible for pathogenic bacterial infection due to the particular mating behaviour on the water containing pathogens from faecal excretions, which may reduce the laying performance in goose (Jiang *et al.* 2011, Yang *et al.* 2012). The ovarian steroid hormones regulate follicular development, atresia and ovulation in avian species (Johnson 2012, 2015). The deleterious effect of pathogens on follicular growth may involve the direct action on follicles, since TLRs recognising the pathogens are present in follicles (Subedi *et al.* 2007, Michailidis *et al.* 2010, Brownlie & Allan 2011). However there is little information about dynamic changes of steroid hormones and TLRs expression levels in responses to bacterial infection in the goose ovarian follicles.

TLRs have been identified as the key components of innate immune response in animal, providing the first line of defence against potential pathogens (Aderem & Ulevitch 2000, Akira *et al.* 2001, Subedi *et al.* 2007, Michailidis *et al.* 2010, 2014, Brownlie & Allan 2011). To date, 10 types of TLRs, including TLRs 1A, 1B, 2A, 2B, 3, 4, 5, 7, 15 and 21, have been identified in chicken (Subedi *et al.* 2007, Temperley *et al.* 2008, Michailidis *et al.* 2010). It has been reported that some TLRs were expressed in the chicken ovary, and were stimulated by LPS or *Salmonella enteritidis* (SE), indicating a TLR-mediated immune response mechanism (Subedi *et al.* 2007, Michailidis *et al.* 2010). Moreover, TLRs have been hypothesised to take part in follicular development and ovulation (Woods *et al.* 2009, Kannaki *et al.* 2011). *In vitro* experiments suggest that LPS or SE treatment lead to the differential regulation of TLRs based on the stage of follicular maturation, with the largest follicle granulosa cells having the most rapid immune response and the undifferentiated granulosa cells being more sensitive to LPS/SE-induced apoptosis (Woods *et al.* 2009, Wang *et al.* 2014). Additionally,

the granulosa cell proliferation and steroidogenesis are inhibited during the SE infection (Tsai *et al.* 2010, Wang *et al.* 2014).

The LPS, derived from gram-negative bacteria, is usually used to mimic bacterial infections. It is now well established that LPS not only induces the host immune response, but also has a detrimental effect on cell function (Cronin *et al.* 2012, Saut *et al.* 2014, Oguejiofor *et al.* 2015). The LPS-mediated ovarian inflammatory response, reduced steroidogenesis, increased cell apoptosis, and inhibited follicular and luteal growth in mammals have been elaborated in previous studies (Herath *et al.* 2007, Herzog *et al.* 2012, Bromfield & Sheldon 2013, Luttgenau *et al.* 2016b). Furthermore, these procedures are mediated via TLRs 2 and 4 pathways (Shimada *et al.* 2008, Bromfield & Sheldon 2011, Price & Sheldon 2013, Price *et al.* 2013, Glynn *et al.* 2014, Luttgenau *et al.* 2016a). Recently the synergistic effect of TLRs 4 and 5 to initiate the innate immune responses against *Escherichia coli* (*E. coli*) infection has been described in mouse epididymal epithelial cells (Cheng *et al.* 2016), which indicates a potential interaction among TLRs. Moreover, the other TLRs, including TLRs 1A, 7, 15 and 21, could be induced by LPS in chicken Sertoli cells (Michailidis *et al.* 2014). It is proposed that in addition to TLRs 2 and 4, LPS could induce the expression of the other TLRs in the ovarian follicles, which perturbs ovarian function.

Therefore this study was initially designed to determine the expression pattern of TLR families, and then to investigate time course effect of LPS treatment on the expression levels of TLR families and steroidogenesis in the goose ovarian follicles.

Materials and methods

Experimental birds

The experiment was conducted at the Yangzhou Goosing Agricultural Science and Technology Co., Ltd (Jiangsu, China; longitude 119.39° and latitude 32.59°). The geese of age 540 days approximately at peak laying were placed in pens containing artificial water pool, slate floor bed and living area. The living area was under low light intensity at night. The others were kept under natural photoperiod. The birds were provided with feed and water *ad libitum*. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Jiangsu Academy of Agricultural Sciences, and performed according to the guide for animal care and use of laboratory animals.

Experimental design

A stock solution of LPS was prepared by dissolving LPS from *E. coli* 055:B5 (Sigma) in Dulbecco's phosphate buffer (D-PBS; Gibco). The changes in the expression levels of TLRs in follicles were examined at 0, 6, 12, 24 and 36 h after the injection

of LPS (1.5 mg/kg BW). Ten geese were selected immediately after LPS injection for 0 h as the control, while five geese were selected at other time point. All experimental birds were slaughtered 8 h after oviposition by cervical dislocation, and the blood samples were collected. In all experiments, the white and yellowish follicles, and granulosa and theca layers of hierarchical follicles were collected. The follicular tissues from the control group were used to analyse the changes in the TLRs mRNA expression during follicular growth. The spleen from the control group was used to determine the expression of TLRs as a positive control.

Blood collection

The blood sample (2 mL) from each bird was collected through the wing venipuncture, and immediately placed in ice-cold heparinised tubes just before slaughtering. The serum was separated by centrifugation at 4°C, diluted three times and stored at -20°C for hormonal measurements.

Laying behaviour determination

The methods for determining the laying behaviour were used with modifications (Qin *et al.* 2013). Briefly, a flock of laying Yangzhou geese, about 2000, were placed in 5 connected pens. Each pen was equipped with 5 nest boxes which were equipped with a monitoring system connected to a computer. Video monitoring of laying behaviour and the presence of an egg in the nest box helped determine the timing of oviposition.

Tissue collection

The theca and granulosa layers were isolated according to the procedure as described previously (Porter *et al.* 1989, Sechman *et al.* 2014) with minor modifications. Briefly, the ovaries were collected within 5 min after slaughtering the birds and placed in ice-cold D-PBS. The white and yellowish follicles, and the first largest to the fifth largest hierarchical follicles (F1–F5), were isolated from each ovary using ophthalmic scissors. The white and yellowish follicles were snap-frozen in liquid nitrogen for further analysis. The outer connective tissue surrounding the surface of each F1–F5 follicle was removed. The yolk was then drained through incisions made in the follicular wall. The follicle was inverted and the granulosa layer was teased loose. Then, the granulosa layer and the remaining theca layer tissues were collected separately and snap-frozen in liquid nitrogen. The theca and granulosa layers of hierarchical follicles that became an irregular ellipse or circle in shape and deep yellow in appearance after LPS treatment for 24 or 36 h (Fig. 3) were not isolated, since the yolks were gelatinous and could not be drained out, which resulted in failure to invert the follicles. Thus, these denatured hierarchical follicles (DF) were directly snap-frozen in liquid nitrogen.

Hormone assays

Plasma E2 and P4 concentrations were measured by paramagnetic particle and chemiluminescent immunoassay

Table 1 Primer pairs used for RT-PCR and real-time PCR.

Target gene	Primer sequence (5' → 3')	Accession number	Product size	Annealing temperature
<i>β-Actin</i>	TGACGCAGATCATGTTTGAGA GCAGAGCGTAGCCCTCATAG	M26111	159	57/60
<i>TLR1A</i>	CTAACTGATGTTCCAAA CATTAAGTCAAGCTCTG	XM_013185964.1	271	57
<i>TLR1B</i>	GGCACCAGGCTACAAAATCTG AAGGGAGTGTCAAGCGAAGG	XM_013185965.1	323	57
<i>TLR2A</i>	GAAAGTGGACTAGGTTCCGTTG GCAATGAAGCCCAAGCAC	JN982474	261	60
<i>TLR2B</i>	ATAAGCATAAGAAGGGTAGA CAATCCCAGGCACAGTAGC	JQ687404	196	60
<i>TLR3</i>	TAATCTGGCTATTTCTCCT CCAAAGTCGTGCTAAATTA	KC292270	160	57
<i>TLR4</i>	GGTGCCACATCCATACAAT TAGGTCAGTCAGAGAGGATA	HQ436371	173	60
<i>TLR5</i>	GGGGAAGAACATATCAACA GATCACTAAAGTACCTGCT	KC845942	139	60
<i>TLR7</i>	CAACCTTTCCCAGAGCATT TCCTCAGCCTAACATACCG	JQ910168	191	60
<i>TLR15</i>	ATATGAGGCTCAGACGAAG GGTAGTCCACAGCGGTTAG	JQ014619	100	60
<i>TLR21</i>	TGTGCTTCTACCTTTCACCG TGGTACTTCAGCCGCCAGT	JN573269	106	60

(Access Estradiol Assay and Access Progesterone Assay, respectively, Beckman Coulter, Inc.) using the Access Immunoassay Systems (Unicel Dxl 800 Access, Beckman Coulter, Inc.) according to the manufacturer's recommendations. The sensitivity of the E2 and P4 assays was 20 pg/mL and 0.10 ng/mL respectively. The total imprecision of E2 assay was ≤12% at concentrations ≥120 pg/mL. For P4 assay, the imprecision of within run and between run was ≤12% and ≤10% respectively.

RNA isolation, RT-PCR and real-time PCR

Total RNA was isolated from the white and yellowish follicles, granulosa and theca layers of F1–F5, and DF using the RNeasy Pure Tissue Kit (Qiagen, Beijing, China) according to the manufacturer's protocol. All RNA samples were treated with DNase I digestion to avoid genomic DNA contamination.

For the geese in the control group and after LPS treatment for 24 and 36 h, all RNA samples were reverse transcribed individually using PrimeScript RT Master Mix (TaKaRa). For the other geese, RNA samples of the granulosa layer or theca layer from each goose were mixed with equal concentrations, respectively, and were reverse transcribed. The cDNA samples were stored at –20°C until use.

RT-PCR was performed to examine the expression of *TLRs* mRNAs in the spleen, white and yellowish follicles, and theca and granulosa layers. The resulting PCR products were sequenced to confirm the correspondence sequences in GenBank.

RT-PCR and real-time PCR assessment was performed using 40 cycles (95°C for 15 s, 57/60°C for 30 s and 72°C for 30 s). The specific primers (Table 1) used for *TLRs* were designed using Primer Premier 5 software (PREMIER Biosoft International). For real-time PCR, each sample was assayed in triplicate with 0.6 µL (10 mM) of each primer, 1 µL of cDNA

and 10 µL FastStart Universal SYBR Green Master (ROX; Roche Diagnostics) in a total reaction volume of 20 µL. The data for real-time PCR were analysed by the $2^{-\Delta\Delta CT}$ method to calculate the relative level of mRNA in each sample using *β-actin* as the housekeeping gene.

Figure 1 Transcriptional profiling of TLRs in goose ovary. (M) Marker; (N) negative control; (P) positive control (Spleen); (GL) granulosa layer; (TL) theca layer; (WF) white follicle; (YF) yellowish follicle.

Figure 2 The mRNA expression levels of TLRs in follicles and during follicular growth. (WF) White follicle; (YF) yellowish follicle; (GL) granulosa layer; (TL) theca layer; (F1) the first largest follicle; (F2) the second largest follicle; (F3) the third largest follicle; (F4) the fourth largest follicle; (F5) the fifth largest follicle. Values with the different lower case letters in GL and/or TL at the same gene are significantly different ($P < 0.05$).

Statistical analysis

Statistical analyses were carried out using the SPSS statistical software program (version 13.0; SPSS). The data were analysed for normality using the Kolmogorov–Smirnov goodness-of-fit test. They were transformed to logarithms if not normally distributed, and re-tested for normality before analysis. If still not normalised, the data were tested using the equivalent nonparametric test. The results are expressed as the mean $\pm$ S.E.M., and the differences were regarded as significant at $P < 0.05$.

To analyse the differential expression among the different follicular tissues, the linear mixed model was carried out with comparisons using the Bonferroni correction. The model included the fixed effect of follicular tissue and random effects of goose and different follicular hierarchy. For the expression levels of TLRs in the hierarchical follicles during follicular growth and LPS-treated follicles at different time points, and plasma E2 and P4 concentrations, one-way ANOVA along with Duncan's multiple range test was applied. The expression levels between the theca or granulosa layer in control group and DF, and between theca or granulosa layer at 24 h after LPS treatment and DF were analysed using independent-

samples *t*-test. The Mann–Whitney *U*-test or Kruskal–Wallis *H*-test was used when transformed data were not normally distributed.

Results

Transcriptional profiling of TLRs in the goose ovary

Expression of TLRs in the goose ovarian follicles determined by RT-PCR is presented in Fig. 1. All 10 reported TLRs in hen, namely TLRs 1A, 1B, 2A, 2B, 3, 4, 5, 7, 15 and 21, were expressed in the white and yellowish follicles, and granulosa and theca layers of hierarchical follicles.

Expression of TLRs during follicular growth

The expression levels of TLRs in the follicles and during follicular growth are presented in Fig. 2. All 10 members of the TLRs were differentially expressed among the white and yellowish follicles, and granulosa and theca

Figure 3 The changes in the morphology of ovary follicles after 0, 6, 12, 24 and 36 h challenge with LPS.

layers of hierarchical follicles. For the prehierarchical follicles, the expression levels of *TLRs 1A*, *1B*, *5* and *15* were higher ($P < 0.05$) in the white follicles than in the yellowish follicles. Regarding the hierarchical follicles, the expression levels of *TLRs 3*, *5*, *15* and *21* were higher ($P < 0.05$) in the granulosa layer than in the theca layer, while those of *TLRs 2A*, *4* and *7* were lower ($P < 0.05$).

The mRNA levels of *TLRs 2A* and *15* in the theca layer and *TLR15* in the granulosa layer exhibited a tendency to increase with follicular growth. The *TLR2A* mRNA expression in the F2 and *TLR15* mRNA expression in the F1 were higher than those of F4 and F5 ($P < 0.05$), while the *TLR15* mRNA expression in the F2 was higher than that in the F3, F4 and F5 ($P < 0.05$).

Time course effect of LPS on follicle morphology

The time course effect of LPS treatment on follicle morphology is shown in Fig. 3. The morphology and appearance colouration of ovarian follicles did not change at 0, 6 and 12 h after LPS treatment. However, at 24 h, the hierarchical follicles of three geese became an irregular ellipse or circle in shape and deep yellow in colour. Moreover, the yolks were gelatinous. The similar morphological changes were found in all five experimental geese at 36 h after LPS injection.

Figure 4 Time course effect of LPS on plasma P4 and E2 concentrations. Values with the different capitals are significantly different ($P < 0.05$).

Time course effect of LPS on plasma P4 and E2 concentrations

The time course effect of LPS treatment on plasma P4 and E2 concentrations is shown in Fig. 4. Plasma P4 and E2 concentrations were decreased at 12 h ($P < 0.05$), and then gradually decreased until 36 h after LPS treatment ($P < 0.05$).

Time course effect of LPS on TLRs mRNA expression in the follicles

Quantification of the mRNA levels of the expressed *TLRs* were performed in the prehierarchical and hierarchical follicles after 0, 6, 12, 24 or 36 h stimulation with LPS by real-time PCR analysis (Fig. 5). The theca and granulosa layers in DF were not isolated, and hence the time course of mRNA expression in *TLRs* was not analysed in the theca and granulosa layers at 36 h after LPS stimulation.

Prehierarchical follicles

Compared with the control (LPS treatment for 0 h), the expression levels of six *TLRs* were upregulated following LPS stimulation in the white follicles. *TLR15* mRNA expression was increased during the 6–24 h period before declining at 36 h ($P < 0.05$). The expression levels of both *TLRs 2A* and *4* were increased ($P < 0.05$) at 12 and 24 h, while *TLR5*, *TLR7* and *TLR3* mRNA expression was increased ($P < 0.05$) only at 6, 12 and 24 h respectively. However *TLR21* and *TLR1A* mRNA expression was decreased at 36 h, and during the 24–36 h period ($P < 0.05$) respectively. There was no significant effect of LPS on *TLRs 1B* and *2B* mRNA expression at all time points.

Regarding the yellowish follicles, stimulation with LPS (1.5 ng/kg BW) resulted in a significant upregulation of eight *TLR* genes, but all of them were not stimulated at 36 h. The expression levels of *TLRs 1B* and *15* increased during the 6–24 h period ($P < 0.05$). *TLR1A* showed higher expression at 6 and 12 h ($P < 0.05$). The mRNA levels of *TLRs 2A*, *4* and *5* mRNA were all increased at 12 and 24 h ($P < 0.05$), while *TLR2B* and *TLR7* mRNA expression was increased at 6 h and at 12 h ($P < 0.05$) respectively. However LPS stimulation did not significantly influence the expression levels of *TLRs 3* and *21*.

Hierarchical follicles

The expression levels of eight *TLRs* were stimulated, while only *TLR3* mRNA expression was decreased at 24 h after LPS stimulation in the granulosa layer ($P < 0.05$). The expression levels of *TLRs 7* and *15* were increased during 6–24 h period ($P < 0.05$). *TLR21*, and *TLRs 2A* and *4* showed higher expression during the 6–12 h period and during the 12–24 h period ($P < 0.05$) respectively, while *TLR1A*, *TLR1B* and *TLR5*

Figure 5 Time course effect of LPS on TLRs mRNA expression in follicles. (WF) White follicle; (YF) yellowish follicle; (GL) granulosa layer; (TL) theca layer. Values with the different lower case letters among different time periods of LPS at the same gene and the same tissue are significantly different ($P < 0.05$).

mRNA expression was increased only at 6, 12 and 24 h ($P < 0.05$) respectively.

In the theca layer, out of ten expressed genes, seven *TLRs* were stimulated by LPS treatment. The expression levels of *TLRs* 3, 4, 7 and 15 were all increased during the 6–24 h period ($P < 0.05$). *TLR1A* and *TLR2A* mRNA expression was increased during the 6–12 h period and during the 12–24 h period ($P < 0.05$) respectively, while *TLR2B* mRNA expression was increased only at 12 h ($P < 0.05$).

Differential expression of TLRs between DF and hierarchical follicles

Differential expression of *TLRs* between DF and hierarchical follicles is presented in Fig. 5. The expression levels of four *TLRs*, namely *TLRs* 2A, 4, 7 and 15, were higher in the DF than in the granulosa layer, while only the *TLR3* mRNA expression was lower. The remaining five *TLRs* showed similar expression. Except for *TLR21*,

the others showed higher expression levels in the DF than in the theca layer ($P < 0.05$).

The *TLR1A* expression level was higher in the DF than in the granulosa layer at 24 h after LPS stimulation, while *TLRs* 3 and 5 expression levels were lower in the DF. The expression levels of four *TLRs*, namely *TLRs* 1A, 1B, 5 and 21, were higher in the DF than in the theca layer after LPS treatment for 24 h, while the remaining *TLRs* showed similar expression levels.

Discussion

To our knowledge, this study firstly elaborated the dynamic changes in the *TLRs* expression levels and steroidogenesis in avian ovarian follicles during LPS infection. Surprisingly, the 10 types of *TLRs*, reported in hen so far, were all expressed in white and yellowish follicles, and granulosa and theca layers in goose ovarian follicles. Moreover, except *TLRs* 2 and 4, the expression levels of all other *TLRs* increased after LPS treatment. Additionally, it was found that the ovarian follicle morphology was altered after long-time LPS stimulation along with stimulated *TLRs* expression, which was negatively associated with plasma P4 and E2 concentrations.

TLRs can recognise a broad spectrum of pathogens. For example, *TLRs* 2A, 3, 4, 5 and 7 recognise the peptidoglycans of gram-positive bacteria, double-stranded RNA, lipopolysaccharides of gram-negative bacteria, bacterial flagellin and single-stranded RNA respectively (Honstetter et al. 2004, Brownlie & Allan 2011). *TLR15* may recognise the bacterial protease and heat stable substance (Nerren et al. 2010, Boyd et al. 2012), and *TLR21* may recognise CpG DNA of bacteria (Kestra et al. 2010). Inconsistent with previous study in chicken elucidating that *TLRs* 2, 4, 5 and 7 express in the theca layer and *TLRs* 4 and 5 express in the granulosa layer (Subedi et al. 2007), the results of this study show that all 10 *TLRs* were expressed in the white and yellowish follicles, and granulosa and theca layers of the hierarchical follicles, which further confirm that *TLR* expression in one species may not be predictive of what will occur in another species (Akira et al. 2001, Yoshimura 2015). Due to the particular mating behaviours in water, it would be challenging for the ovary to recognise the unexpected pathogenic microorganisms ascending from the oviduct (Jiang et al. 2011, Yang et al. 2012). Thus, we suggest that the ovary could effectively recognise invading microorganisms, even if the behavioural habits increase the risk of bacterial infection. Moreover, there are different sensitive sites for pathogens-associated molecular patterns (PAMPs) among different follicular tissues resulting from differential expression of *TLRs*.

The data of this study show that *TLRs* 2A and 15 mRNA expression in the theca layer and *TLR15* mRNA expression in the granulosa layer increased with follicular growth, which is inconsistent with previous

study that the expression levels of *TLRs* 4 and 5 in the theca layer and that of *TLR5* in the granulosa layer increased with the follicular growth (Subedi et al. 2007). This is possibly due to changes in the pathogen involved (Michailidis et al. 2014).

In accordance with the previous studies in chicken reproductive tissues (Ozoe et al. 2009, Woods et al. 2009, Das et al. 2011, Zhang et al. 2012, Ariyadi et al. 2014), the expression levels of *TLRs* 2 and 4 was upregulated by LPS stimulation in the goose follicles. Moreover, this study, for the first time, showed that LPS stimulated the expression of other *TLRs*. It has been established that LPS is recognised by *TLR4*; however whether the LPS is also recognised by *TLR2* or not remains ambiguous. Some reports show that *TLR2* mediates LPS-induced cellular signalling (Fukui et al. 2001, Heumann & Roger 2002, Spiller et al. 2007), but the others suggest that LPS activates cellular signalling through *TLR4*, not *TLR2* (Hirschfeld et al. 2000, Tapping et al. 2000). Although the reason for increase in other *TLRs* mRNA expression after LPS treatment is not known, a recent study reported that *TLRs* 4 and 5 synergistically initiate the innate immune responses to *E. coli* infection in mouse epididymal epithelial cells (Cheng et al. 2016), indicating a potential interaction among *TLRs*. The interaction of *TLRs* and their PAMPs induces an inflammatory response, which involves the host immune (Michailidis et al. 2014). Thus, we suggest that not only exogenous LPS could be directly recognised by *TLR4* to initiate an innate immune response, but it could trigger the expression of other *TLRs* to strengthen this process.

The mature granulosa cells have a more significant and progressively stronger immune response after LPS/SE infection (Woods et al. 2009, Wang et al. 2014). In this study, after LPS treatment for 6 h, the expression levels of two *TLR* genes in white follicles, two genes in yellowish follicles, four genes in granulosa layer and five genes in theca layer increased. Furthermore, the longest time duration of stimulated *TLRs* expression occurs in the theca layer, followed by that in the granulosa layer, yellowish follicles and white follicles, which indicates that the hierarchical follicles have a more significant and progressively stronger immune response than prehierarchal follicles.

Previous studies showed that only *TLR3* mRNA expression in the ovary and testis exhibited a tendency to decrease following SE infection (Michailidis et al. 2010, Anastasiadou et al. 2011), but it was not affected in the vagina and chicken Sertoli cells (Michailidis et al. 2011, 2014). In our study, only *TLRs* 1A and 21 in the white follicles and *TLR3* in the granulosa layer exhibited a tendency of decrease in the expression level with prolonged LPS treatment. It is not surprising that the bacterial LPS infection did not stimulate *TLRs* 1A, 3 and 21 expressions because of their specific PAMPs (Schwarz et al. 2007, Chen et al. 2013); however these genes expression pattern in the other ovarian tissues

were stimulated by LPS in our study. We suggest that the effect of bacterial LPS on *TLRs* 1, 3 and 21 expression is dependent on the avian reproductive tissue or cell types; however, this molecular mechanism needs to be further studied.

In mammals, LPS enhances apoptosis of corpus luteum and reduces the levels of oestradiol and progesterone in ovary (Price *et al.* 2013, Magata *et al.* 2014a,b, Luttgenau *et al.* 2016b), which may lead to compromised reproductive performance. Similarly, it is reported that the chicken granulosa cells induce suppression of cell proliferation (Tsai *et al.* 2010) and P4 synthesis (Wang *et al.* 2014) during SE infection. In this study, preovulatory follicle, especially the larger follicle, morphology is altered after prolonged LPS stimulation, indicating the induction of follicular atresia. Although the preovulatory follicles are generally resistant to become atretic (Johnson 2003, 2015), increased blood flow with follicular growth (Scanes *et al.* 1982) may result in an accumulation of large amounts of circulating LPS to larger rather than smaller follicles. Furthermore, the circulating E2 and P4 levels are gradually decreased (Fig. 4), which indicates that steroidogenesis of follicle is perturbed. To our knowledge, this is the first study developing an *in vivo* model to investigate the time course effect of lipopolysaccharide on *TLRs* expression and steroidogenesis of ovary in birds.

The undifferentiated granulosa cells were more sensitive to LPS/SE-induced apoptosis than differentiated granulosa cells in the chicken, which reflects the susceptibility of granulosa cells at the early stages of maturation to undergo apoptosis in response to select pathogenic stimuli (Woods *et al.* 2009, Wang *et al.* 2014). In our study, although the most types of *TLRs* expression levels in the white and yellowish follicles were stimulated by LPS treatment, expectedly, they were not continuously stimulated until 36 h. We suggest that long-time exogenic LPS stimulation increased prehierarchical granulosa cell apoptosis, and then decreased the *TLRs* signalling function. Moreover, the growth and maturation of the ovarian follicles is associated with the differentiation of granulosa cells, which occurs before the selection of yellowish follicle into the preovulatory hierarchy (Johnson & Woods 2009, Johnson 2015). Thus, bacterial infection might inhibit the development of prehierarchical follicles into the hierarchical follicles.

It was surprising that the expression of four genes in GL and nine genes in TL was significantly lower than those in the DF in this study, which indicates that these damaged follicles have more capacity to recognise PAMPs and induce *TLR* signalling. Furthermore, the circulating E2 and P4 levels gradually decreased at 12h after LPS treatment. The avian reproductive tract has the ability to express proinflammatory cytokines and chemokines, which are essential for the activation, differentiation and control of the immune system

(Abdelsalam *et al.* 2011, Nii *et al.* 2011, Zhang *et al.* 2012, Sonoda *et al.* 2013, Michailidis *et al.* 2014). Moreover, the LPS stimulation resulted in the increased influx of heterophil-like cells and T cells in the theca layers of follicles (Abdelsalam *et al.* 2011). It is likely that the innate immune capabilities in the ovarian follicular cells modulate their endocrine function (Herath *et al.* 2007). Thus, together with the results of plasma P4 and E2 concentrations and follicle morphology, we propose that the immune function of the avian ovarian follicles gradually grows for responding to pathogen infection, while steroidogenesis gradually declines during LPS infection. However this continuous inflammatory reaction might damage the follicular structure.

It has been reported that through *TLRs* 2 and 4, LPS reduced steroidogenesis in mammalian ovaries (Bromfield & Sheldon 2011, Price & Sheldon 2013, Price *et al.* 2013, Glynn *et al.* 2014, Luttgenau *et al.* 2016a). Likewise, LPS stimulated the expression levels of *TLRs* 2 and 4, accompanied by lowered steroidogenesis in birds (Woods *et al.* 2009, Wang *et al.* 2014). The results presented in this study showed that the expression pattern of *TLRs* 2A and 4 genes was similar to the other genes, such as *TLR15* in the white follicles; *TLRs* 1B, 5 and 15 in the yellowish follicles; *TLRs* 7 and 15 in the granulosa layer and *TLRs* 1A, 2B, 3, 7 and 15 in the theca layer, which were negatively correlated with the kinetics of plasma P4 and E2 concentrations during LPS infection. In the avian nonhierarchical follicles, E2 synthesis is higher than that in the hierarchical follicles, whereas P4 synthesis remains opposite (Sechman *et al.* 2014). Meanwhile, the granulosa layer produces mainly P4, while the theca layer produces E2 (Johnson *et al.* 2002, Woods & Johnson 2005, Johnson & Woods 2009, Johnson 2015). Thus, we suggested that not only *TLRs* 2 and 4, but also *TLRs* 1A, 1B, 3, 5, 7, and 15 may play important roles in LPS-inhibited steroidogenesis after interacting with their ligands in birds. Moreover, these effects are dependent on the stage of follicular maturation and type of follicular cells.

In conclusion, the ten *TLRs* are expressed in the Chinese goose ovarian follicles, and LPS infection triggers innate immune response not only through *TLR4*, but also by stimulating the expression of the other *TLRs*. Moreover, LPS alters the follicular structure and reduces the steroidogenesis, which might explain the phenomenon that increased microorganism concentrations are associated with decreased laying performance in goose. The data presented in this study also suggest that LPS may inhibit the steroidogenesis through induced *TLRs* expression in the ovarian follicles. In addition, these effects may be dependent on the stage of follicle maturation and the type of follicular cells. Our data expand previous understanding and provide novel insight into the function of *TLRs* in the avian ovary follicles.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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