Apoptosis induction and release of inflammatory cytokines in the oviduct of egg-laying hens experimentally infected with H9N2 avian influenza virus

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A B S T R A C T
The H9N2 subtype avian influenza virus (AIV) can cause serious damage to the reproductive tract of egg-laying hens, leading to severe egg-drop and poor egg shell quality. However, previous studies in relation to the oviductal-dysfunction resulted from this agent have not clearly been elucidated. In this study, apoptosis and pathologic changes in the oviducts of egg-laying hens caused by H9N2 AIV were evaluated. To understand the immune response in the pathogenic processes, 30-week old specific pathogen free (SPF) egg-laying hens inoculated with H9N2 subtype of AIV through combined intra-ocular and intra-nasal routes. H9N2 AIV infection resulted in oviducal lesions, triggered apoptosis and expression of immune related genes accompanied with infiltration of CD3+CD8+ and CD3+CD8α+ cells. Significant tissue damage and apoptosis were observed in the five oviducal parts (infundibulum, magnum, isthmus, uterus and vagina) at 5 days post-inoculation (dpi). Furthermore, immune-related genes, including chicken TLR3 (7, 21), MDA5, IL-2, IFN-β, CXCL1, CXCL2, CCL2, XCR1 and CCR5 showed variation in the egg-laying hens infected with H9N2 AIV. Notably, mRNA expression of IFN-α was suppressed during the infection. These results show distinct expression patterns of inflammatory cytokines and chemokines amongst segments of the oviduct. Differential gene expression of inflammatory cytokines and lymphocytes aggregation occurring in oviducts may initiate the infected tissue in response to virus replication which may eventually lead to excessive cellular apoptosis and tissue damage.

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1. Introduction

H9N2 subtype avian influenza (AI) is an acute infectious disease caused by influenza A virus, a member of the family Orthomyxoviridae belonging to the low pathogenic avian influenza virus (AIV) that has been widespread in domestic poultry in Asian countries since the mid-1990s (Alexander and Capua, 2008). It has been shown that egg-laying hens infected with H9N2 AIV show symptoms of mild bleeding in the respiratory, digestive and reproductive tracts (Pantin-Jackwood et al., 2012). H9N2 AIV can also have
pronounced effects on the immune systems of infected chickens leading to immune suppression and subsequent secondary infection (Bano et al., 2003).

It is well recognized that host immunological and genetic factors play an important role in the pathogenesis of influenza A viruses in mammals. Previous studies have shown that lethal infections caused by AIH are a consequence of complex interactions between the virus and host immune systems which include excessive inflammatory response in the form of a cytokine storm initiated inside the infected cells or tissues following viral replication resulting in excessive cellular apoptosis and tissue damage (Kash et al., 2014; Manvell et al., 2003). In vitro studies have suggested that H9N2 AIV can initiate strong innate, immune and pro-inflammatory responses (TLR3, TLR7, MDA5, TNF-α and CCL5) in addition to cellular apoptosis in human tracheobronchial and intestinal epithelial cells (Qu et al., 2012; Xing et al., 2011). Apoptosis and induction of inflammatory cytokines have also been identified in immortal DF-1 cells by H9N2 AIV infection (Liu et al., 2015; Shao et al., 2014). It is known that apoptosis and hyper induction of inflammatory cytokines caused by avian influenza virus in the respiratory and digestive tracts are major causes of respiratory and digestive failure (Auewarakul, 2009; Bi et al., 2010). The oviduct is an important organ of egg-laying hens that can produce each element of eggs and is, therefore, a valid target for investigating the mechanism of dysfunction caused by H9N2 AIV.

Our previous study identified Sα2,3 receptors which are recognized and adsorbed by the hemagglutinin of avian influenza virus, to be were widely distributed along the oviduct in hens (Wang et al., 2013a). These findings provide the possibility that the oviduct is one of the potential target organs for the virus. In contrast to the extensive work performed in mammalian organisms following influenza virus infection, much remains to be determined about the underlying mechanism of viral infection in the oviducts of egg-laying hens. Currently, there is no known report concerning the activation of immune related genes, induction of apoptosis and lymphocytes recruitment of chicken oviducts infected with H9N2 AIV. The results presented in this study showed altered expression of immune-related genes in five segments of the oviduct. MDA5, TLR21 were the main sensors, whilst IFN-α gene expression was suppressed by H9N2 AIV infection. Verification of apoptosis and virus replication, differential regulation of immune related genes expression and infiltration of CD3+CD4+ and CD3+CD8α+ cells in oviducts, all of which may play critical roles in dysfunction of the oviduct were investigated.

2. Materials and methods

2.1. Animals and virus

Thirty 30-week old SPF White Leghorn egg-laying hens were supplied by Yang Ling Green Biological Engineering Company. Animals were randomly divided into five groups, 6 animals for each experimental group as follows; one group was used as a mock control group and sacrificed at 0 h before infection; the remaining four groups were infected with H9N2 AIV (A/chicken/Shaanxi/11/2012) (Wang et al., 2013a,b) through the combined intra-ocular and intra-nasal routes with a total dose of 10⁸ median embryo infective doses, and animals sacrificed at 1 day post-infection (dpi), 3 dpi, 5 dpi, 7 dpi. Prior to the current study all hens were laying regularly. All hens were maintained under uniform standard management conditions with 15 h light and 9 h dark. Hens were housed individually in laying cages, provided with free access to feed and water. Individual laying cycles were monitored by the daily timing of ovisposition. The experiment was performed in labs of BSL2 and conducted in accordance with the rules of the Animal Ethics Monitoring Committee and Animal Welfare Committee of Shaanxi Province, China.

2.2. Collection of samples

The oviducts along with the five parts (infundibulum, magnum, isthmus, uterus and vagina) from mock and experimental groups were collected immediately and placed on ice. The collected oviduct tissues were then used as follows: three oviducts of each group were collected for total RNA extraction, three were fixed in 4% phosphate buffered formalin for the histological analysis by HE staining, TUNEL staining, nucleoprotein (NP) staining and immunofluorescence detection of CD3+, CD4+ and CD8α+ cells.

2.3. Evaluation of viral loads

The colonization of H9N2 AIV in oviducts was examined by SYBR green-based real-time PCR assays. We chose the magnum for examination as it is the most functional and widest segment of the five parts. Primers targeted to the M gene of H9N2 AIV, forward: 5′-TTCTAACGGAGTGCAAAAC-3′(47–65), reverse: 5′-AAGGCTCTACGCTGCA GTCC-3′(275–256) were used for PCR analysis. To perform real-time PCR, viral RNA was extracted from the magnus tissue (0.1 g) then reverse transcribed into cDNA as described in Section 2.8. The cDNA was further subcloned into the PDM⁺TK19-T vector and then transformed into DH5α E. coli. The recombinant plasmid was identified by EcoRI and SalI digestions. Serial 10-fold dilutions of positive recombinated plasmid were applied as a positive quantitative template to establish the standard curve from 10¹ to 10⁸ copies/μL. The real-time PCR reactions (20 μL) contained 2 μL of template RNA samples or a known concentration of standard plasmids, 10 μL of SYBR Green Reaction Mix, 7 μL of RNase Free ddH₂O and 0.5 μL of each primer. The reaction was performed at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s. All amplifications were performed in triplicate. The obtained cycle threshold (Ct) values were plotted against the amount of RNA copy number to the standard curve.

2.4. Indirect immunofluorescence detection of virus nucleoprotein in oviduct

To gain further understanding of virus replication, indirect immunofluorescence detection of virus nucleoprotein in
ovoidal segments was carried out. The formalin-fixed paraffin-embedded tissues sections were deparaffinized with xylene for 8 min then rehydrated through a graded series of alcohol to xylol 100%, 95%, 90%, 80%, and 70% for 3 min at each step. Tissues sections were then blocked for non-specific binding with 5% bovine serum albumin (BSA) in phosphate buffer saline (PBS) for 40 min at 37 °C. After removing blocking solution, sections were incubated overnight at 4 °C with 1 μg/ml mouse anti avian monoclonal antibody specific for nucleoprotein of AIV (Virostat, USA) then stained with anti-mouse specific secondary antibodies labeled with CY5 fluorescent dyes (Santa Cruz, USA). After incubation for 40 min at 37 °C, sections were washed three times with PBS and finally counterstained with Hoechst 33342 (Sigma, USA) for 5 min. After three times wash with PBS, all slides were observed by laser scanning confocal microscope (NIKON, Japan).

2.5. Histological examination

To evaluate the pathological lesions in the reproductive tract of layers, histological examination by hematoxylin and eosin (HE) staining was carried out. Samples collected from each group were cut into small pieces. Tissue sections were deparaffinized with xylene for 8 min then rehydrated through a graded series of alcohol to xylol 100%, 95%, 90%, 80%, and 70% for 3 min at each step, then processed for histology by routine procedures and stained with HE for the histological analysis.

2.6. Apoptosis detection by TUNEL staining

The apoptotic events post-infection were examined by one-step measurement of terminal deoxynucleotidyl transferase mediated d-UTP biotin nick end labeling (TUNEL) Tissue sections were deparaffinized and rehydrated through an alcohol gradient then rinsed in deionized water. The following steps corresponded to the standard operation staining procedures provided by manufacturer (Vazyme Biotech, Nanjing, China).

2.7. Caspase-3 enzyme activity detection

A total 0.1 g of oviduct tissue sample was cut into small pieces, and added to 1 ml of RIPA lysis buffer and homogenised using glass pestle and mortar on ice. The tissue homogenate was transferred into 1.5 ml centrifuge tube and incubated for 5 min. The homogenate was centrifuged at 20,000 rpm for 10 min and the supernatants were extracted into pre-cooled centrifuge tubes. The reaction system (contained 2 μl of supernatant) of caspase-3 enzyme activity was referring to the detection kit (KeyGEN BioTECH, Nanjing, China) and the concentration of caspase-3 enzyme was determined by Bradford Protein Assay Kit (Beyotime Institute of Biotechnology, Nanjing, China).

2.8. Quantitation of mRNA expression of cytokine, chemokine, TLRs and MDA5

Real-time, quantitative RT-PCR was used to estimate the transcripts of cytokines, chemokines, TLRs and MDA5 genes. Total RNA was extracted from individual tissue samples by using TRizol reagent (Takara Biotechnology, Japan). Concentration and purity of RNA preparations was determined by spectrophotometry at A260 versus A280. Each RNA sample was reverse transcribed with EasyScript First-Strand cDNA Synthesis SuperMix (TransGen biotech, China) and the resulting cDNA stored at −20 °C until required.

RT-PCR reactions were performed with equal amounts of cDNA samples from all egg-laying hens and the amplification of various mRNA with specific primers were carried out using a thermal cycler (Bio-Rad, America). Primers were designed for all the genes (shown in Table 1) by using Primer Premier5 software (http://www.premierbiosoft.com/primer design/index.html). The specificity of each primer was checked using the NCBI blast program. All primers were commercially synthesized (Invitrogen, Shanghai, China) and the PCR products were sequenced to assess the specificity of individual primers. Cytokine, chemokine, MDA5 and TLRs gene expression levels in tissues were normalized to those of the housekeeping gene chicken β-actin. The results of real-time PCR were quantified by the comparative threshold analysis after deductions of data from uninfected chickens.

2.9. Immunoﬂuorescence detection of lymphocytes in magnum

Alterations of the T lymphocyte subpopulations during the infection of H9N2 in magnum were investigated by immunofluorescent detection. The dewaxing and rehydrating process as well as BSA treatment was performed as describe in Section 2.4. After removing the blocking solution, sections were incubated for 1 h at 37 °C with 1 μg/ml mouse monoclonal antibody specific for chicken CD markers, FITC conjugated CD3, CY5 conjugated CD4, or RPE labeled CD8α (Southernbiotech, USA) to detect CD3CD4 and CD3CD8α glycoprotein of lymphocytes. After incubation, sections were washed three times with PBS and counterstained with Hoechst 33342 (Sigma, USA) for 5 min. After three washes with PBS, slides were observed using a laser scanning confocal microscope (NIKON, Japan).

2.10. Statistical analysis

The data are presented as the means ± SEM of three independent experiments with three replicates per experiment. Analysis of normalized TLRs, MDA5, cytokine, and chemokine values were carried out using one-way analysis of variance with Dunnett’s post-test using GraphPad Prism version 5.00 for Windows according to Sundaresan’s method (Sundaresan et al., 2008). While t-test was used for caspase-3 enzyme activity analysis as there were only two groups (Day 0 and Day 5) of each oviduct segment. P values less than 0.05, 0.01, 0.001 were considered to be statistically significant.

3. Results

3.1. Viral loads in oviduct

Absolute quantitative detection of virus load by real-time quantitative PCR demonstrated that the pathogen is
Table 1
Sequences of primer pairs used for real-time PCR.

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<th>Genes</th>
<th>Primer sequences</th>
<th>Accession no.</th>
<th>Location</th>
<th>Product size</th>
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<td>TLR7</td>
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<tr>
<td>TLR21</td>
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<td>NM_001193638.1</td>
<td>457–535</td>
<td>79</td>
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<td>NM_205518.1</td>
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capable of replication in oviducts. The detection of magnum showed that the virus load peaked at 5 dpi, whilst there was a decrease in virus load at 7 dpi (data not shown). We further detected the virus replication in five segments of oviduct at 5 dpi and found that there were some differences in five functional parts. The magnum showed the highest amount of virus load (3062.58 copies/μl), followed by the uterus (1356.17 copies/μl), isthmus (1047.81 copies/μl), vagina (800.604 copies/μl) and infundibulum (639.50 copies/μl) (Fig. 1). No virus was found in healthy oviducts.

3.2. Virus distribution along the oviductal segments

The distribution of virus NP protein at 5 dpi was determined by immunofluorescence assay. The results further indicated that H9N2 AIV had spread through blood circulation and the virus is capable of replication in the oviduct. In the infundibulum, the magnum, isthmus and uterus, positive staining was observed in the glandular epithelial cell. In the vagina, the NP proteins were observed in the stroma cells of the lamia propria (Fig. 2). As presented, the NP proteins were observed in the lamia propria but not the mucous layer. In addition, the magnum and uterus showed the strongest positive-staining amongst the five oviductal segments.

3.3. Symptoms and histological changes in oviducts

Symptoms of the infected chickens were observed from the third day of inoculation. Infected chickens were depressed and showed signs of respiratory symptoms. They were consuming less food and water and producing yellow-white, viscous loose stools. Infected chickens were characterized by laying deformed or soft-shelled eggs which on necropsy were discovered to have follicles that were hemorrhagic, congestive or dissolved with some oviducts showing signs of edema and seeping out of white jelly like material.

Histopathological changes were detected at 5 dpi. In magnum and isthmus, some glandular epithelial cells in lamia propria showed degeneration, necrocytosis or fallen off. The mucosal epithelial cell layer became thinner, with cells showing signs of degeneration, necrocytosis or had fallen off. In the uterus, the mucosal epithelial layer also appeared to be degenerated with signs of necrocytosis or had fallen off and the cell numbers in the lamia propria

**Fig. 1.** Virus load of H9N2 AIV in magnum at 5 days post-inoculation (dpi). Primers target to M gene of H9N2 AIV were used to detect the virus replication in five oviductal segments with three replications according to our constructed standard curve whose correlation coefficients (R2) were 0.9901 with a slope value of −3.5023 (y = −3.5023x + 41.207).
were decreased. In the vagina, the mucosal epithelial cells also showed cellular degeneration, necrocytosis and edema or fluid collection within tissues. In addition, scattered lymphocytes were found in the lamina propria of the infundibulum and uterus. Bleeding in the vagina and tissue hyperemia was also observed (Fig. 3).

3.4. Apoptosis detection in infection

TUNEL detection, caspase-3 enzyme activity examination was employed to detect apoptosis in infected layers of oviducts at 5 dpi. TUNEL detection showed H9N2 AIV could induce cellular apoptosis in the lamina propria of the infundibulum, isthmus, uterus and vagina (Fig. 4), whilst in the magnum the cells of both the lamia propria and mucosa displayed signs of apoptosis. Caspase-3 enzyme activities of each of the experimental groups were more than double of the control group, whilst in the magnum, a 3.71-fold up-regulation of caspase-3 was observed (Fig. 5). Caspase-3 enzyme activity examined using the TUNEL assay confirmed H9N2 AIV induced apoptosis in the oviducts of hens. Moreover, apoptosis was significant observed in the magnum and uterus.

3.5. mRNA expression changes of toll like receptors, MDA5, cytokine, and chemokine

Toll like receptor (TLR3/TLR7/TLR21) mRNAs were examined in the oviducts of infected hens. The mRNA expressions of TLRs in five parts of the oviduct were shown to vary. TLR3 was slightly decreased in the first 3 days of infection and was slightly increased at 7 dpi in five segments of oviduct (Fig. 6A). The mRNA expression of TLR7 in five oviductal segments were down-regulated at 1 dpi (0.52, 0.87, 0.41, 0.60, 0.71-fold, respectively), whilst at the other three examined time points, the mRNA expression of TLR7 were up-regulated in each of the five segments (Fig. 6B). As observed for the mRNA expression pattern of TLR7, the mRNA expression of TLR21 was also down-regulated at 1 dpi, whilst being up-regulated in the subsequent measured time points. In the infundibulum, the mRNA expression of TLR21 showed different ranges of
Fig. 3. Pathologic changes of oviduct in H9N2-infected hens. Oviduct sections were from virus-free and H9N2-infected hens, pathological changes was observation by HE staining (400×). (a) Infiltration of lymphocytes; (b) mucosal epithelial cell layer became thinner; mucosal epithelial cell showed degeneration, necrocytosis or fallen off; (c) interstitial tissue edema; (d) glands edema or dissolved. Five oviductal segments from virus-free hens were served as negative control.

Fig. 4. TUNEL staining of oviduct in H9N2-infected hens. Tissues of oviduct were collected at 5 dpi. Nucleus of oviduct sections from virus-free and H9N2-infected hens were stained with Hoechst 33342, red colour indicates the positive staining of apoptotic cells (400×). Oviductal segments from virus-free hens were served as negative control. (For interpretation of the color information in this figure legend, the reader is referred to the web version of the article.)
up-regulation at the four measurement points (Fig. 6C). In general, the mRNA expression of TLR7 and TLR21 was shown to be location specific as it was excessively expressed in the upper two segments of the oviduct. A similar changing trend was shown for both TLR7 and TLR21 mRNA levels in the other three parts of the oviduct (infundibulum, magnum, vagina) which may suggest synergistic effects of TLR7 and TLR21 in response to H9N2 infection (Fig. 6B and C). Another intracellular pattern recognition receptor (PRRs), MDA5, was up-regulated with fluctuating mRNA levels with the highest levels observed at 42.17 and 44.5-fold induction in the magnum and isthmus at 3 dpi respectively, whilst in the vagina, a stable slight increase was maintained between 2.12 and 2.63-fold expression. Amongst the four innate immune genes, MDA5 showed the highest mRNA expression level followed by TLR21, TLR7, and TLR3.

The cytokines mRNA expression examined in this study included IL-2, IFN-α, and IFN-β. These genes did not display similar roles in resisting H9N2 AIV infection. The mRNA expressions of IL-2 were up-regulated in all segments of the oviduct with the ranges shown to be different (Fig. 7A). Relative to the lower three segments, a more drastically modulation was observed in the upper two parts, especially in magnum. The mRNA expression of IL-2 in magnum reached its peak level at 3 dpi with 84.54-fold increase. IFN-α and IFN-β belong to type I interferon which defend organisms from viral attack. The mRNA expression level of IFN-α was significantly decreased by around 0.06–0.65-fold at different time points in the five segments (Fig. 7B). IFN-β was significantly increased in five parts of the oviduct, especially in the magnum where it reached the peak level at 5 dpi with 75.83-fold increase (Fig. 7C).

We also detected mRNA expression levels of chemokines and associated receptors, including CXCL1 (also known as K60), CXCL2 (also known as CAF/IL-8), CXCL1, XCR1 and CCR5. The mRNA expression of CXCL1 (Fig. 8A) increased in five parts of the oviduct during the whole infection process. The magnum also showed the highest level of expression reaching a peak level with 58.20-fold induction at 5 dpi. Like CXCL1, the mRNA expression level of CXCL2 reached its peak at 5 dpi in magnum (Fig. 8B). The up-regulated expression fold change and the time to peak level may reflect the synergistic effects of the two orthologues of human CXCL8. Both XCL1, also named lymphotactin, and its receptor XCR1 were up-regulated by H9N2 infection. The expression level of XCL1 was nearly twice of that for XCR1 in the infundibulum and magnum, and the gene expression fold changes of them in the lower three segments remained relatively stable (Fig. 8C and D). The chemokine receptor CCR5 also showed an up-regulated trend during the infection process and was

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**Fig. 5.** Caspase-3 enzyme activity detection in oviducts of H9N2-infected hens. Total protein was extracted from infected and uninfected chickens at 5 dpi (n = 3 per group) with A/Chicken/shaanxi/2012 (H9N2). Caspase-3 enzyme activity was detected using caspase-3 enzyme activity detection kit. Each column represents the fold change of caspase-3 enzyme activity by the comparison to the data of uninfected chickens. Data represents the mean ± SEM of three chickens, paired t-test was used for statistical comparison. *P < 0.05, **P < 0.01.

**Fig. 6.** TLRs, MDA5 mRNA expression in oviducts of hens infected with H9N2 AIV. Total RNA was extracted from infected and uninfected hens (n = 3 per group) with H9N2 AIV/A(Chicken/shaanxi/2012). TLRs were quantified using primers specific for chicken TLR3, TLR7, TLR21, MDA5 and SYBR Green-based real-time PCR. Data represents the mean ± SEM of three hens. Panels A, B, C and D depict results from infundibulum, magnum, isthmus, uterus and vagina of TLR3, TLR7, TLR21 and MDA5. Statistical analysis was performed by the comparison of data of uninfected chickens. *P < 0.05, **P < 0.01, ***P < 0.001.
up-regulated at the mRNA level in the infundibulum (1.23–1.36-fold) and the isthmus (2.52–4.95-fold), but in the other three segments, the fold changes of mRNA expression level appeared to fluctuate (Fig. 8E).

### 3.6. Dynamic changes of CD3+CD4+, CD3+CD8α+ cells in the oviduct

Based on the results of the highest sensibility to virus replication and the highest level of cytokine mRNA expression, the magnum was used for further investigation. The aggregation of CD3+, CD4+, and CD8α+ cells in the magnum was greater in the treatment groups than in the control group, and the double positive stain of CD3+CD4+ and CD3+CD8α+ cells can be clearly observed (Figs. 9 and 10). The distribution of CD3+CD4+ and CD3+CD8α+ cells was sporadic in the mock group and slightly aggregated at 24 h after infection before reaching a peak at the fifth day. The distribution of CD3+CD4+ and CD3+CD8α+ cells in the lamina propria and the mucosal epithelium of the magnum were different with regard to duration of infection. The CD3+CD8α+ cells were more aggregated than that of CD3+CD4+ cells in the bottom layer of the mucosal epithelium and in lamina propria. The infiltration and migration of CD3+CD8α+ cells were more remarkable than that of CD3+CD4+ cells.
Fig. 8. The mRNA expression of chemokine and chemokine receptors in oviducts of H9N2 AIV infected hens. Induction of chemokines and chemokine receptors using total RNA (Fig. 6) was quantified using primers specific for chicken cytokines and SYBR Green-based real-time polymerase chain reaction. Data represent the mean ± SEM of three hens. Panels A, B, C, D and E depict results from infundibulum, magnum, isthmus, uterus and vagina of CXCL1, CXCL2, XCL1, XCR1 and CCR5. Statistical analysis was performed by the comparison of data of uninfected hens. *P < 0.05, **P < 0.01, ***P < 0.001.

4. Discussion

Avian H9N2 viruses have been widespread in domestic poultry in Asian countries since the mid-1990s with AIV infections causing mortality ranging from 5 to 30%. Chickens infected with H9N2 AIV have mild to severe respiratory signs which include edema of the head and face (Kim et al., 2006). Our in vivo experiments have verified that H9N2 AIV causes tissue lesions in the oviducts of egg-laying hens and results in the reduction in both egg production and quality. This is a microcosm of domestic poultry when H9N2 AIV outbreaks. The study successfully infected egg-laying hens with H9N2 AIV and the virus survived and was replicated in the oviducts, especially in the magnum. It has been observed that H9N2 AIV infection can induce significant clinical symptoms, pathologic changes, apoptosis and activation of immune responses in hens which may contribute to oviductal dysfunction.

It was well known that influenza A virus, especially the H5 subtype influenza virus, can infect multiple organs and cells in mammals and birds. The research group of Silva has shown that HPAIV can cause lesions in the reproductive tract at 36–72 h after inoculation, although the injuries caused by different strains were variable (Silva et al., 2013). In this study, we found congestion of blood vessels along the oviduct and invasion of inflammatory cells in infundibular section. In addition, the mucosa of the magnum showed necrosis of the epithelial villus that had fallen off and showed glandular necrosis. In practice, hens infected with H9N2 AIV lead to decrease in the number and quality of eggs. We measured the eggshell and albumin quality in another study in which the result suggested that H9N2 AIV also caused eggshell thinning and decreases in the expression of ovalbumin (OVA) and calcium-binding protein D28 K (CaBP-D28k) (data not shown). From the perspective of pathological changes and apoptosis in this study, we postulated that the lesions occurred in the glandular and mucosa aroused by H9N2 AIV infection in the five segments may be responsible for the poor performance in egg-laying hens.

The increasing activity of caspase-3 and positive TUNEL staining indicated that the oviductal tissues were undergoing cellular apoptosis in the early and late stages of H9N2 AIV infection. Virus load checking confirmed viral replication in the oviductal segments and positive staining of the AIV NP protein in the oviducts of egg-laying hens identified the infected cells were glandular epithelium and stromal cells in lamina propria. Apoptosis was consistently observed in the five oviductal segments. It is also noteworthy that the mucosal layer of the magnus underwent severe apoptosis but was not found to have NP protein of the virus. We speculate that the richest SAα2, 3 receptor distribution on mucous epithelium cells of the magnum was one of the reasons for the viral pathogenicity and the regulation of immune system.
We further examined whether PRRs, inflammatory cytokines and chemokines in each of the five sections of oviduct in response to H9N2 infection. We measured the mRNA expression levels of three TLRs (TLR3/7/21), MDA5, three cytokines (IL-2, IFN-α, IFN-β), three chemokines (CXCL11, CXCL12, XCL1) and two chemokine receptors (XCR1, CCR5).

TLRs and MDA5 belong to different PRRs which recognize viral nucleic acids are central to host antiviral defences. The activation of PRRs by viral nucleic acids triggers the expression of innate antiviral genes such as interferon (Chen et al., 2013). In the present study, TLR3 was slightly decreased in the early stage of infection and was slightly increased in late stage that may trigger the down-regulation of IFN-α mRNA expression, as TLR3 plays two-faced role in the induction of antivirus IFN-α or IFN-β in different situation (Okahira et al., 2005; Kogut et al., 2005). The expression of both TLR7 and TLR21 were shown to be location specific as they were excessively expressed in the upper two segments, and only slightly changed in the lower three parts of the oviduct. The mRNA expressions of IL-2, and IFN-β were also shown to be up-regulated as

Fig. 9. CD3+CD4+ cells infiltration in magnum of H9N2 AIV infected hens. Magnum of oviduct collected at 0, 1, 3, 5 and 7 dpi. Magnum sections from virus-free and H9N2 AIV infected hens, nucleus was stained by Hoechst 33342 (A, B, 200×). CD3+ cells in magnum were detected by FITC-conjugated mouse-chicken monoclonal antibody. CD4+ cells in magnum were detected by CY5-conjugated mouse-chicken monoclonal antibody. Green colour indicates the positive staining of CD3+ cells, red color indicates the positive staining of CD4+ cells. The double positive stain of CD3+, CD4+ indicates that these cells express both of CD3+, CD4+ glycoproteins. Panel A depict results from five oviductal segments of virus-free hens, Panel B depict results from five oviductal segments of virus-infected hens. (For interpretation of the color information in this figure legend, the reader is referred to the web version of the article.)
These results demonstrate that H9N2 AIV can induce an immune response in the local oviduct. The differential expression of PRRs may suggest that MDA5 was the main sensor. The up-regulated of MDA5 gene expression coincided with the up-regulation of IFN-β gene expression as MDA5 can be employed by chicken cells including DF-1 fibroblasts and HD-11 macrophage for sense AIV, and strongly active the chicken IFN-β promoter (Liniger et al., 2012). An interesting finding was that the mRNA expression of IFN-α was down-regulated in five segments during H9N2 AIV infection in chickens. Chicken IFN-α is a potential prevention and therapeutic antiviral agent that inhibits H9N2 AIV replication and induces antiviral interferon simulated genes (ISGs) expression. Lacking the mRNA expression of IFN-α may attribute to the down-regulation of TLRs and may be benefit for the virus surviving strategy. The down-regulation of TLR3 gene, and the differential mRNA expression of IFN-α/β may imply the different involvement of these two chicken IFNs in antiviral response. The passive gene expression role played by TLR3 and IFN-α may indicate that the lacking of them would contribute to the virus replication.

Fig. 10. CD3⁺CD8α⁺ cells infiltration in magnum of H9N2 AIV infected hens. Magnum of oviduct collected at 0, 1, 3, 5 and 7 dpi. Magnum sections from virus-free and H9N2 AIV infected hens, nucleus was stained by Hoechst 33342 (A, B, 200×). CD3⁺ cells in magnum were detected by FITC-conjugated mouse-chicken monoclonal antibody. CD4⁺ cells in magnum were detected by CY5-conjugated mouse-chicken monoclonal antibody. Green colour indicates the positive staining of CD3⁺ cells, salmon color indicates the positive staining of CD8α⁺ cells. The double positive stain of CD3⁺, CD8α⁺ indicates that these cells express both of CD3⁺, CD8α⁺ glycoproteins. Panel A depict results from five oviductal segments of virus-free hens, Panel B depict results from five oviductal segments of virus-infected hens. (For interpretation of the color information in this figure legend, the reader is referred to the web version of the article.)
Chemokines are evolutionarily conserved and mediate cellular responses to stress. Chemokine receptors guide immune cells to migrate to specific organs during health or disease. Chemokine systems regulate this process by directing the circulation of immune cells and their recruitment to sites of infection. The mRNA expression of CXCL1 and CXCL2 can be induced in lymphocytes, heterophils and oviduct epithelial cells (Chappell et al., 2009; Shini et al., 2009). XCL1 is produced by T, NK and NKT cells during infectious and inflammatory responses, whereas XCR1, the receptor of XCL1, is expressed by a dendritic cell subpopulation. The XCL1-XCR1 axis plays an important role in dendritic cell mediated cytotoxic immune response (Lei and Takahama, 2012). CCR5 is a receptor for the macrophage inflammatory protein MIP family (Hughes et al., 2007). The up-regulation in the expression trends of chemokines and chemokine receptors may potentially attract immune cells and involved in modulating cellular immunity.

Cell-mediated immune responses play an important role in early virus infection with the cytotoxic T lymphocyte response efficiently reducing virus shedding (Seo and Webster, 2001). Intestinal epithelial cells infected with influenza virus present viral antigens to antigen-specific CD8+ cytotoxic T lymphocytes and the induction of virus-specific cytotoxic T lymphocytes as a basis for the development of broadly protective influenza vaccines (Nguyen et al., 1998; Hillaire et al., 2011). It is reported that T lymphocytes express CD3 and CD8α/CD8ββα on cell surface. The surface of NK cells can express CD8α but cannot express CD3. CD3 is expressed in cytoplasm of NK cells (Gobel et al., 1994). Therefore, T cells and NK cells cannot be distinguished in this study using immunofluorescent staining. Recently, a novel chicken CD3+CD4+ blood population with NK cell like feature has been identified, we cannot assure that whether the CD4+ cells were T cells or not (Neulen et al., 2015). Besides that, CD3+CD8α+ cells are located and infiltrated more in the bottom of the mucosal epithelium than CD3+CD4+ cells. The migration and aggregation of CD3+CD8α+ cells appeared faster than that of CD3+CD4+ cells suggesting the high mRNA expression of CXCL11, CXCL2, XCL1, XCR1, CCR5 are related to the infiltration of these two population of cells. In addition, the faster migration and aggregation of CD3+CD8α+ cells are hypothesized to mediate the viral clearance and may also be responsible for the exacerbated tissue lesions.

In conclusion, the altered expression of inflammatory factors and apoptosis induced by H9N2 AIV infection may play an important role in the severe local damage found in the oviducts of egg-laying hens. Virus replication, apoptosis and inflammatory immune response showed that the magnus was the most sensitive segments of the oviduct to react to H9N2 AIV. Different modulation of immune-related gene expression reflected that chicken TLRs and MDA5 were initiated by H9N2 AIV and it may provide a link between innate and cell-mediated immunity. Further studies are needed to determine the mechanisms of apoptosis and immune responses on infected hens’ oviductal cell lines in vitro.

Author contribution

Jingyu Wang and Chao Tang performed the majority of experiments and involved in manuscript preparation. Quizhen Wang, Ruqiao Li, Zhanli Chen, Xueying Han and Jing Wang participated part of the experiments. Jingyu Wang conceived the study, participate in its design and coordination. Xingang Xu revised the manuscript. All authors read and approved the final manuscript.

Conflict of interest

There is no conflict of interest of any authors in relation to the submission.

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