High ambient humidity aggravates ammonia-induced respiratory mucosal inflammation by eliciting Th1/Th2 imbalance and NF-κB pathway activation in laying hens

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ABSTRACT Ammonia (**NH_3**) is an irritant and harmful gas. Its accumulation in the poultry house poses detrimental effects on the respiratory mucosal system of birds. In this process, the relative humidity of the poultry house also plays an important role in potentiating the adverse effects of NH_3 on the respiratory status of birds, causing severe physiological consequences. In this study, the combined effects of NH_3 and humidity on the respiratory mucosal barrier of laying hens was studied. The gene expression of tight junction proteins, mucin, inflammatory cytokines secreted by Th1/Th2 cells, and proteins related to the Nuclear factor-κB (**NF-κB**) signaling pathway were detected by qRT-PCR. In addition, the contents of mucin and secretory immunoglobulin A (**SIgA**) in bronchoalveolar lavage fluid (**BALF**) were determined. The results showed that treatment with NH_3 alone or NH_3 and humidity led to morphological changes in the respiratory tract, decreased the gene expressions of tight junction protein, and increased the expression of mucin. Also, the expression of interleukin-4 (***IL-4***) and *IL-10* were increased, whereas, the expression of interferon-γ (***IFN-γ***) and *IL-2* was decreased in laying hens treated with NH_3 and humidity. Furthermore, the activation of inhibitor kappa B kinase β (***I-KK-β***) and the degradation of inhibitor of NF-κB α (***I-κB-α***) contributed to the activation of the *NF-κB* pathway, such that the downstream genes, cycooxygenase 2 (***COX2***) and inducible nitric oxide synthase (***iNOS***) were significantly increased. In conclusion, NH_3 damaged the mucosal barrier and induced an imbalance in the mucosal immunity, leading to respiratory tract inflammation. Thus, the relative humidity of the environment aggravates the adverse effects of NH_3 in poultry.

Key words: laying hens, ammonia, relative humidity, respiratory tract mucosa, immune function

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INTRODUCTION

Ammonia (**NH_3**) is a harmful gas and an important source of air pollution. Within the environment, NH_3 is largely generated from agricultural production, and about 90% of these emission emanates from livestock and poultry production, such as during poultry manure treatment and feed volatilization (Beusen et al., 2008; Sutton et al., 2013; Groenestein et al., 2019). Under suitable temperature and humidity conditions, the feces, feed residues, and bedding materials in the poultry house are decomposed by microorganisms to produce NH_3. When the distal large intestine of poultry lacks carbohydrates, the bacteria in the intestine can ferment amino acids to obtain energy and produce harmful gases such as NH_3 and hydrogen sulfide (Louis et al., 2014). Under the effect of urease, the uric acid excreted by the kidneys is also decomposed into NH_3 gas and emitted into the environment (Davila et al., 2013). High levels of NH_3 stimulate the eyes and visceral organs, affecting the health and growth of poultry (Wei et al., 2014; Zhang et al., 2015; Naseem and King, 2018). The respiratory system which serves as the conduit for gaseous exchange is vulnerable to the effects of harmful gases. NH_3 can alter the permeability of cell membranes, and then enter the lymph through the blood-air barrier, triggering a mucosal immune response (Zhao et al., 2013). Broilers exposed to high concentrations of NH_3 suffer an increased infiltration of inflammatory substances into

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the respiratory mucosa (Miles et al., 2006). Damaged barriers and disturbed immunity result in impaired resistance to disease. Research has proved that NH_3 adsorbed as dust reduces the resistance of poultry to harmful substances and increases the susceptibility to diseases (Curtis et al., 1975). Therefore, besides the structure and functioning of the respiratory mucosal barrier, the respiratory mucosal immune system is an important consideration in addressing the detrimental effects of NH_3 in poultry.

The respiratory mucosal barrier constitutes a mechanical barrier, chemical barrier, microbial barrier, and immune barrier, which functions to protect the respiratory health of poultry. The stability of tight junction is crucial for the mechanical barrier, and also participates in the body's innate immunity (Sawada, 2013). In the chemical barrier, the main protein, mucin does not only adhere to bacteria but also lubricates the intestinal lumen (Thornton et al., 2008; Johnson, 2011). Certain species of microorganisms in the respiratory tract form a stable microbial barrier to resist and eliminate foreign pathogens. In the mucosal immune system, the humoral immunity dominated by secretory immunoglobulin A (**SIgA**) plays an important role in defense against pathogens (Macpherson et al., 2001; Peterson et al., 2007; Cerutti, 2008). Studies have shown that NH_3 stimulated changes in the cytokines secreted by Th2 and Th17, which in turn aggravated the imbalance of Treg/Th1 and led to tracheal inflammation (Shi et al., 2019). Thus, the changes in cytokine levels are related to the response of T helper cells. The Th1 cells mainly secrete interferon-γ (**IFN-γ**) and interleukin-2 (**IL-2**), which inhibit the differentiation of Th2 cells and promote cellular immunity. The Th2 cells secrete IL-4 and IL-10, which inhibit the differentiation of Th1 cells and promote humoral immunity. Nuclear factor-κB (**NF-κB**) is a nuclear factor involved in the activation of immune cell and inflammatory signaling pathways (Zou et al., 2018). Studies have shown that several inflammatory conditions induced by harmful gas molecules are related to the activated NF-κB pathway, such as the sulfur dioxide (**SO_2**) induced asthma in rats, and the hydrogen sulfide (**H_2S**) induced pneumonia in broilers (Li et al., 2014; Wang et al., 2018). Proinflammatory cytokines, such as tumor necrosis factor-α (**TNF-α**) and IL-1β, can activate the NF-κB signaling pathway. More so, the inflammatory factors induced from the NF-κB pathway elicit the continuous activation of NF-κB via a positive feedback mechanism (Luan et al., 2010; Liu et al., 2017).

In commercial production, various environmental factors can interact to harm the respiratory health of laying hens. Typically, the relative humidity of laying pens is maintained between 50 and 65%. In a high temperature and humidity environment, the walls, bedding materials, and uric acid from the feces are acted on by microorganisms to generate noxious gases including NH_3 (Groot Koerkamp and Bleijenberg, 1998). It was reported that the concentration of NH_3 in the poultry house increased with an increase in humidity, which aggravated the adverse effects of NH_3 on the production performance of livestock and poultry (Weaver and Meijerhof, 1991). The combined effect of humidity and NH_3 not only inhibits the evaporative heat dissipation and respiratory rate of poultry but also damages the physiological environment of the respiratory tract and reduces production performance (Yahav et al., 1995; Yahav et al., 1998). However, the mechanism behind the coeffect of NH_3 and humidity on the health of the respiratory tract in laying hens is not fully understood.

This study examined the detrimental effects of NH_3 and humidity on the respiratory mucosal barrier of laying hens and further investigated the role of Th1/Th2 balance and NF-κB signaling pathway in this process. The blood NH_3 content, morphological changes and gene expressions of tight junction protein, mucin, and NF-κB pathway proteins were examined in the respiratory tract of laying hens.

MATERIALS AND METHODS

All procedures in the study were approved by the Animal Care Committee of Shandong Agricultural University and were performed in accordance with the guidelines for experimental animals of the Ministry of Science and Technology (Beijing, China).

Experimental Animals and Treatment

Hy-Line Brown layers (n = 288) at 53 wk old were used for this study. According to ammonia concentration and relative humidity, birds were randomly divided into four groups: 0 mg/m^3 NH_3 + 55% RH, 60 mg/m^3 NH_3 + 55% RH, 60 mg/m^3 NH_3 + 75% RH, and 60 mg/m^3 NH_3 + 95% RH. All laying hens had free access to feed and water. The light regimen was 13 L:11 D (5 Lux), and the dark period was from 07:00 pm to 06:00 a.m. At the 3rd and 6th wk of the experiment, 8 laying hens were randomly selected per treatment and sacrificed by cervical dislocation. The bronchoalveolar lavage fluid (**BALF**) was collected. Parts of the trachea and lung were preserved in 4% paraformaldehyde for histology. Sections of the trachea and lung tissue were collected and frozen in liquid nitrogen, then stored at −80°C.

Collection of BALF

At the upper half of the trachea, about 3 cm of the trachea was cut open and placed into 3 mL PBS to acquire the lavage. After shaking at 3,000 rpm/min for 5 min and centrifuging at 4°C for 30 min, the supernatant was collected as BALF.

Histological Observation of the Trachea and Lung

After tissue fixation in 4% paraformaldehyde for at least 24 h, the tissues were dehydrated with gradient

Table 1. Gene-specific primer sequences used for gene transcription analyses of chicken.

Gene	GeneBank accession no.	Primer sequences (5'-3')	Orientation	Product size (bp)
GAPDH	NM_204305.2	CTACACACGGACACTTCAAG	Forward	244
		ACAAACATGGGGGCATCAG	Reverse	
Claudin-1	NM_001013611.2	ATGACCAGGTGAAGAAGATGC	Forward	182
		TGCCCAGCCAATGAAGAG	Reverse	
Occludin	XM_046904540.1	GGTTCCTCATCGTCATCCTGCTC	Forward	154
		GCCACGTTCTTCACCCACTCCT	Reverse	
ZO-1	XM_046925214.1	CTTCAGGTGTTTCTCTTCCTCCTC	Forward	131
		CTGTGGTTTCATGGCTGGATC	Reverse	
MUC2	XM_040673077.2	CCCTGGAAGTAGAGGTGACTG	Forward	143
		TGACAAGCCATTGAAGGACA	Reverse	
MUC5AC	XM_040673078.2	AAGACGGCATTTATTTCTCCAC	Forward	244
		TCATTACCAACAAGCCAGTGA	Reverse	
SIgA	S40610.1	AGGGCAATGAGTTCGTCTGT	Forward	114
		TCAGGAGGGTCACTTTGGAG	Reverse	
PIgR	NM_001044644.2	GGATCTGGAAGCCAGCAAT	Forward	123
		GAGCCAGAGCTTTGCTCAGA	Reverse	
TNF-α	XM_046927265.1	GCCCTTCCTGTATACAGATG	Forward	71
		ACACGACAGCCAAGTCAACG	Reverse	
IL-1β	XM_046931582.1	TCTTCTACCGCCTGGACAGC	Forward	145
		TAGGTGGCGATGTTGACCTG	Reverse	
IFN-γ	NM_205149.2	ACCTTCCTGATGGCGTGAAG	Forward	80
		GCGCTGGATTCTCAAGTCGT	Reverse	
IL-2	NM_204153.2	GAACCTCAAGAGTCTTACGGGTCTA	Forward	111
		ACAAAGTTGGTCAGTTCATGGAGA	Reverse	
IL-4	XM_046900385.1	TTGTTTGGGAGAGCCAGCAC	Forward	102
		GACATGGTGCCTTGAGGGAG	Reverse	
IL-10	NM_001004414.4	CAGACCAGCACCAGTCATCA	Forward	162
		TCCCGTTCTCATCCATCTTCTC	Reverse	
NF-κB	XM_046915553.1	TCAACGCAGGACCTAAAGACAT	Forward	106
		GCAGATAGCCAAGTTCAGGATG	Reverse	
I-KK-β	XM_046931637.1	TGATAGCAAGGTGAATGACGCTGTAG	Forward	167
		CGGATGAGGTCGCAAGGCAAC	Reverse	
I-KB-α	NM_001001472.3	CTTCCAGAACAACCTCAGCCAGAC	Forward	92
		CGCAGCCAGCCTTCAGCAG	Reverse	
COX2	XM_046922435.1	TGTCCTTTCACTGCTTTCCAT	Forward	84
		TTCCATTGCTGTGTTTGAGGT	Reverse	
iNOS	NM_204961.2	CCTGGGTTTCAGAAGTGGC	Forward	82
		CCTGGAGGTCCTGGAAGAGT	Reverse	

increased alcohol from 70 to 100%. Then the samples were embedded into paraffin after the rapid clearing process with xylene. Thereafter, samples were sectioned into 5-μm slices and dewaxed with xylene. Rehydration was done with a gradient-reduced alcohol-water blend from 100 to 70%, then the sections were stained with hematoxylin for 2 min and washed with running water. The color was differentiated with 1% HCL solution. Subsequently, the samples were stained with eosin for 3 min. After the dehydration again, the section was dried and sealed with neutral resin. Tissue sections were observed with light microscopy (Nikon, ECLIPSE 80i).

Blood Ammonia Content

The serum ammonia content was determined using a blood ammonia assay kit according to the manufacturer's instruction (Nanjing Jiancheng Bioengineering Institute, China).

Mucin Content in BALF

The content of mucin in BALF was determined according to the sulfuric acid-phenol method described by Dubois et al. (1956).

SIgA Content in BALF

The content of SIgA in BALF was determined by the double antibody sandwich method using the chicken secretory immunoglobulin A (SIgA) ELISA Kits (Mlbio Co., China).

Gene Expressions Analysis by Real-time Quantitative PCR

The mRNA expression level of selected genes in the trachea and lung were analyzed by real-time quantitative PCR. According to the manufacturer's instructions, total RNA was extracted from the trachea and lung using Trizol reagent (Invitrogen, San Diego, CA). The reverse transcription of total RNA into cDNA was performed according to the instruction of the kits (Takara, China). The primers used are given in Table 1. qRT-PCR was performed using TB Green Premix Ex Taq II (Tli RNaseH Plus, TaKaRa) on Applied Biosystem QuantStudio 3 System (Applied Biosystems, Foster City, CA). The relative mRNA levels were calculated according to the $2^{-\triangle\triangle Ct}$ method, and GAPDH was used as the internal reference for normalization.

Statistical Analysis

The data are shown as the mean ± standard error. The effect of ammonia at the same humidity (0 mg/m^3 NH_3 + 55% RH and 60 mg/m^3 NH_3 + 55% RH) and the effect of humidity at the same ammonia concentration (60 mg/m^3 NH_3 + 55% RH, 60 mg/m^3 NH_3 + 75% RH, and 60 mg/m^3 NH_3 + 95% RH) were compared and analyzed, respectively. All data were statistically analyzed using one-way analysis of variance (**ANOVA**) with SAS software (Version 8.1; SAS Institute Inc., Cary, NC). $P < 0.05$ and $P < 0.01$ were considered statistically significant and extremely significant differences, respectively.

RESULTS

The Effect of NH_3 and Humidity on the Content of Ammonia in the Blood

The results of blood ammonia content showed that at the 55% relative humidity, the blood ammonia content of the 60 mg/m^3 NH_3 group increased significantly in the 3rd wk ($P < 0.05$, Figure 1) and 6th wk ($P < 0.01$, Figure 1) compared to 0 mg/m^3 NH_3 group. When the concentration of ammonia was 60 mg/m^3, the blood ammonia content in 60 mg/m^3 + 95% RH group was significantly higher than that in 60 mg/m^3 + 55% RH group ($P < 0.01$, Figure 1) and 60 mg/m^3 + 75% RH group ($P < 0.05$, Figure 1) at the 3rd wk of the experiment. In the 6th wk of the experiment, the content of ammonia in the blood increased significantly with the increase in relative humidity ($P < 0.05$, Figure 1) compared to the 60 mg/m^3 + 55% RH group.

The Effect of NH_3 and Humidity on the Morphological Structure of the Respiratory Tract

To determine whether the ammonia and humidity can damage the tissue structure of the respiratory tract, we observed the results of H&E staining of the trachea and lung. As shown in Figures 2A and 2B, the tissue structure of the trachea and lung in the 0 mg/m^3 NH_3 + 55% RH group was normal. The tracheal cilia were arranged neatly and there was no adhesion. Relatively, the stimulation of 60 mg/m^3 NH_3 resulted in the fall, loss, and adhesion of tracheal cilia in the 60 mg/m^3 NH_3 + 55% RH group (Figures 2A and 2B). At the same time, the blood vessels of the lung were swollen and appeared congested (Figures 2C and 2D). With the increase in relative humidity, the extent of damage and shedding of tracheal cilia increased and the blood vessels of the lung appeared severely congested with edema (Figures 2A−2D). The trachea's mucosa and lung tissues of laying hens in the 60 mg/m^3 NH_3 + 95% RH group showed increased infiltration of inflammatory cells and interstitial space (Figures 2A−2D).

The Effect of NH_3 and Humidity on the Expression of Tight Junction Proteins

The effect of ammonia and humidity on the mRNA expression level of tight junction protein in the respiratory tract is shown in Figure 3. At the 55% relative humidity, 60 mg/m^3 NH_3 significantly downregulated the expression of *ZO-1* in the trachea and *Occludin* in the lungs in the 3rd wk compared to the 0 mg/m^3 NH_3 + 55% RH group ($P < 0.05$, Figures 3C and 3E). At the 6th wk, 60 mg/m^3 NH_3 significantly downregulated the gene expression levels of *Occludin* and *ZO-1* in both trachea and lung tissues compared to the 0 mg/m^3 NH_3 + 55% RH group ($P < 0.05$, Figures 3B, 3C, 3E and 3F). At the 60 mg/m^3 NH_3 concentration, relative to 60 mg/m^3 NH_3 + 55% RH group, 95% relative humidity significantly downregulated the gene expression levels of *Claudin-1* in trachea at the 3rd wk ($P < 0.05$, Figure 3A). At the 6th wk, 60 mg/m^3 NH_3 + 75% RH significantly decreased the gene expression level of *ZO-1* compared to 60 mg/m^3 NH_3 + 55% RH group ($P < 0.05$, Figure 3C). Importantly, 60 mg/m^3 NH_3 + 95% RH significantly

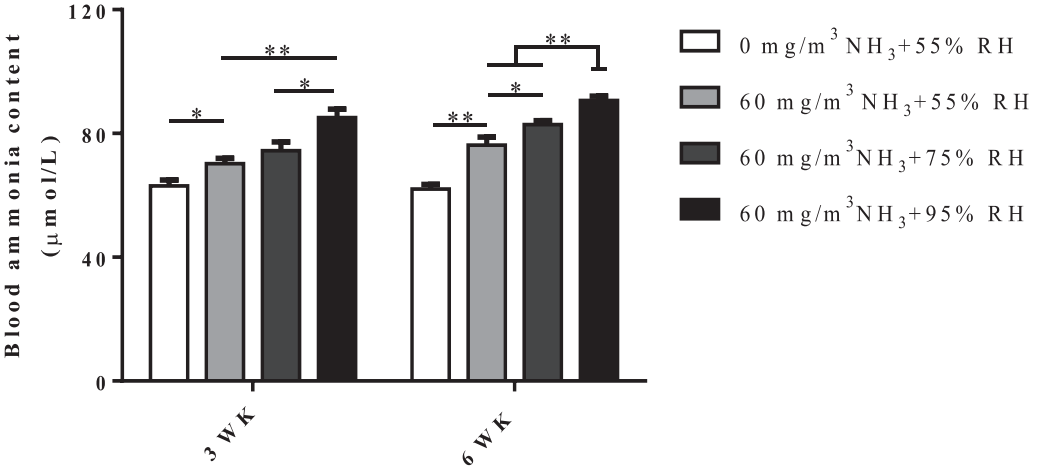

Figure 1. The effect of ammonia and humidity on the blood ammonia content. The data are expressed by the mean ± standard error. * indicates $P < 0.05$; ** indicates $P < 0.01$; n = 8.

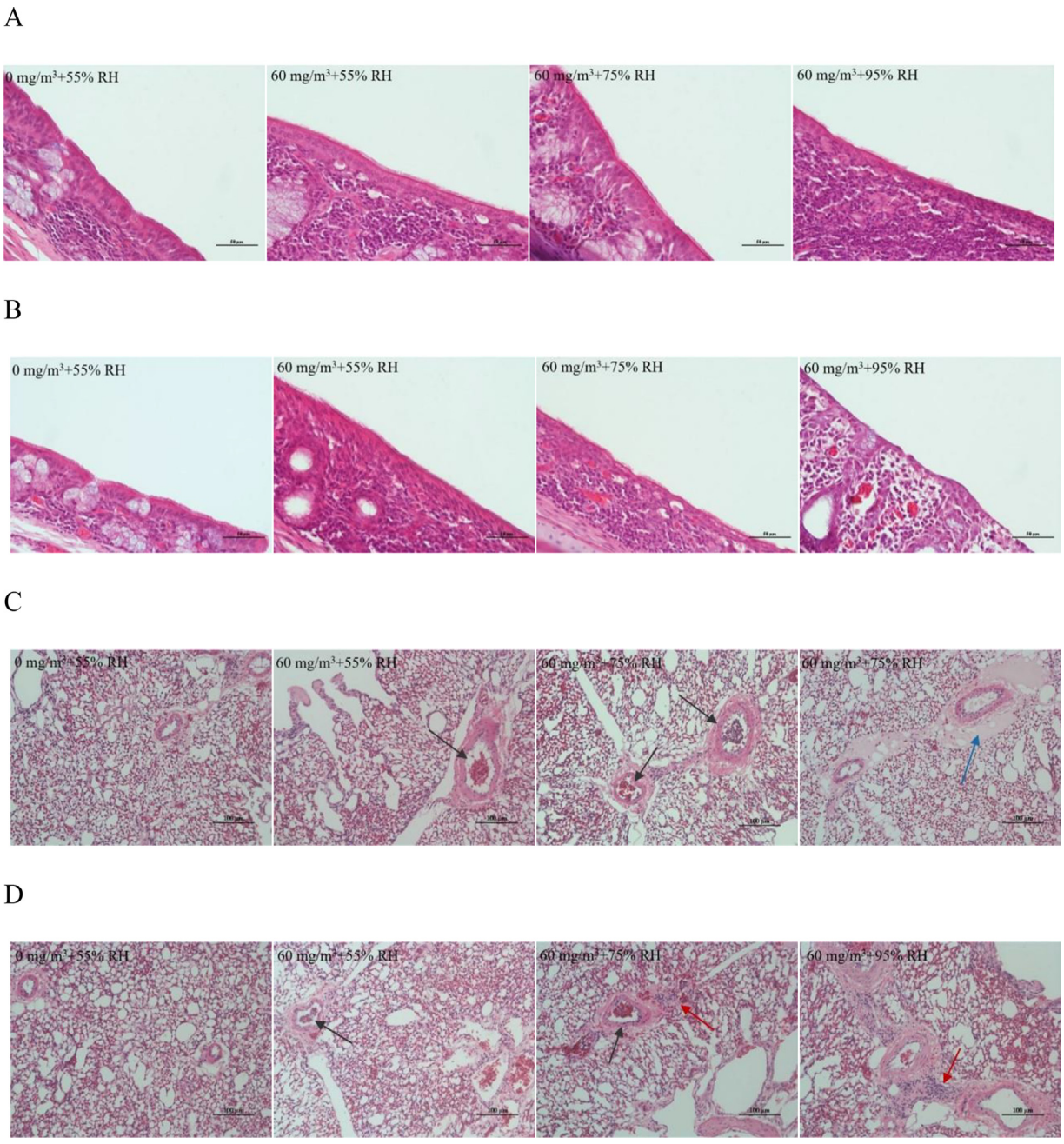

Figure 2. The effect of ammonia and humidity on the structure of the respiratory tract. (A) and (B) show the H&E staining results of the trachea at 3rd and 6th wk, respectively (H&E, × 400). (C) and (D) show the H&E staining results of the lung at 3rd wk and 6th wk, respectively (H&E, × 200). The black arrows point out blood vessel congestion. The blue arrows point out angioedema. However, the red arrows point out inflammatory cell infiltration.

reduced *Claudin-1, Occludin*, and *ZO-1* in the 6th wk.

The Effect of NH_3 and Humidity on the Content and Gene Expression of Mucin

To confirm the influence of NH_3 and humidity on the chemical barrier of the respiratory tract, we detected the content of mucin in BALF. Under the 55% relative humidity, 60 mg/m^3 NH_3 significantly increased the content of mucin in the BALF ($P < 0.01$, Figure 4A). Compared with 60 mg/m^3 NH_3 + 55% RH group, the content of mucin in BALF increased significantly in the 60 mg/m^3 NH_3 + 95% RH group at 6th wk ($P < 0.05$, Figure 4A). The gene expressions of mucin in the trachea and lung were also detected. Compared with 0 mg/m^3 NH_3 + 55% RH group, 60 mg/m^3 NH_3 enhanced the gene expression level of *MUC2* in the trachea in the 6th wk ($P < 0.05$, Figure 4B), and also enhanced the gene expression level of *MUC5AC* in the trachea and lung at 3rd and 6th wk ($P < 0.05$, Figures 4C and 4E). Relative to 60 mg/m^3 NH_3 + 55% RH group, 75 and 95% relative humidity significantly upregulated the gene expression of *MUC5AC* in the trachea in the 3rd wk and in lung tissue at 3rd and 6th wk ($P < 0.05$, Figures 4C and 4E).

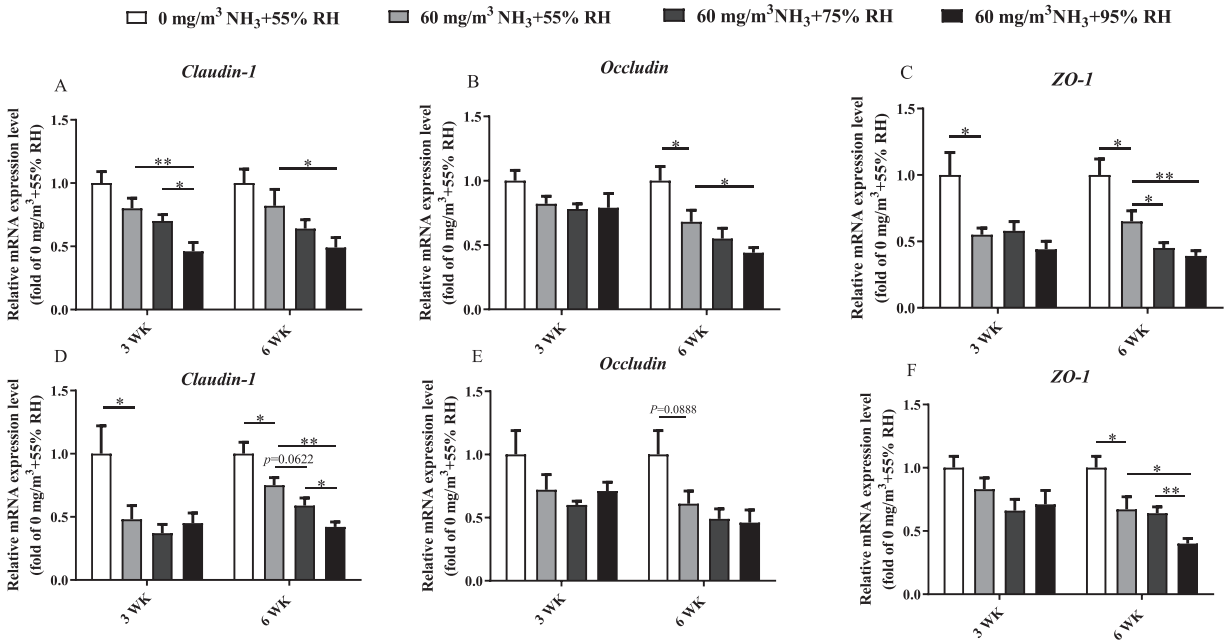

Figure 3. The effect of NH_3 and humidity on the mRNA levels of tight junction proteins. (A), (B), and (C) show the gene expression levels in the tracheal. (D), (E), and (F) show the gene expression levels in the lung. The data were expressed by the mean ± standard error. * indicates $P < 0.05$; ** indicates $P < 0.01$, n = 8.

The Effect of NH_3 and Humidity on the Secretion and Expression of SIgA

SIgA is the most important antibody in the mucosa of the respiratory tract, and its content can reflect the levels of mucosal immune response of the respiratory tract to a certain extent. As shown in Figure 5, the exposure of laying hens to 60 mg/m^3 NH_3 significantly upregulated the gene expression levels of *SIgA* in the trachea ($P < 0.01$) at the 3rd and 6th wk, and also enhanced the gene expression level of *pIgR* in the lung ($P < 0.05$) at the 3rd wk (Figures 5B and 5E). During treatment with 60 mg/m^3 NH_3, the content and gene expression level of *SIgA* in BALF increased with an increase in the relative humidity ($P < 0.05$, Figures 5A and 5B). In 3rd wk, the laying hens treated with 60 mg/m^3 NH_3 + 95% relative humidity showed higher SIgA content and gene expression level in the BALF than those in the 60 mg/m^3

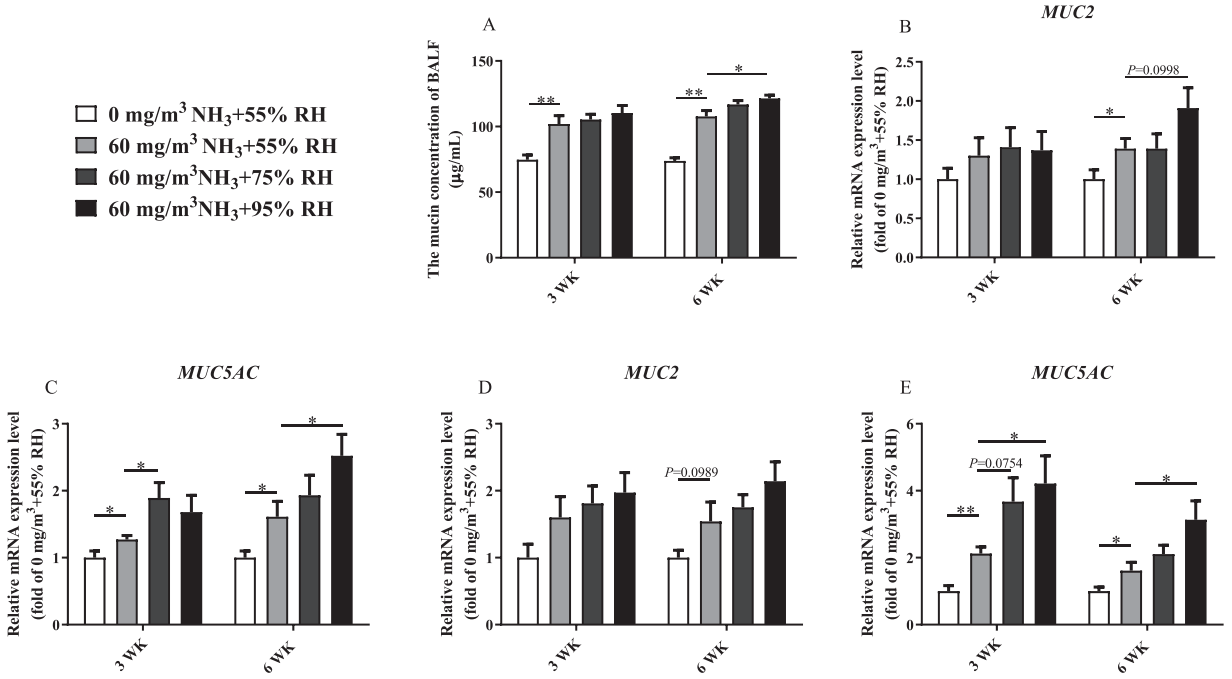

Figure 4. The effect of NH_3 and humidity on the content of mucin and the gene expression levels of *MUC2* and *MUC5AC*. (A) shows the content of mucin in BALF. (B) and (C) show the genes expression levels of *MUC2* and *MUC5AC* in the tracheal. (D) and (E) show the gene expression levels of *MUC2* and *MUC5AC* in the lung. The data are expressed by the mean ± standard error. * indicates $P < 0.05$; ** indicates $P < 0.01$, n = 8.

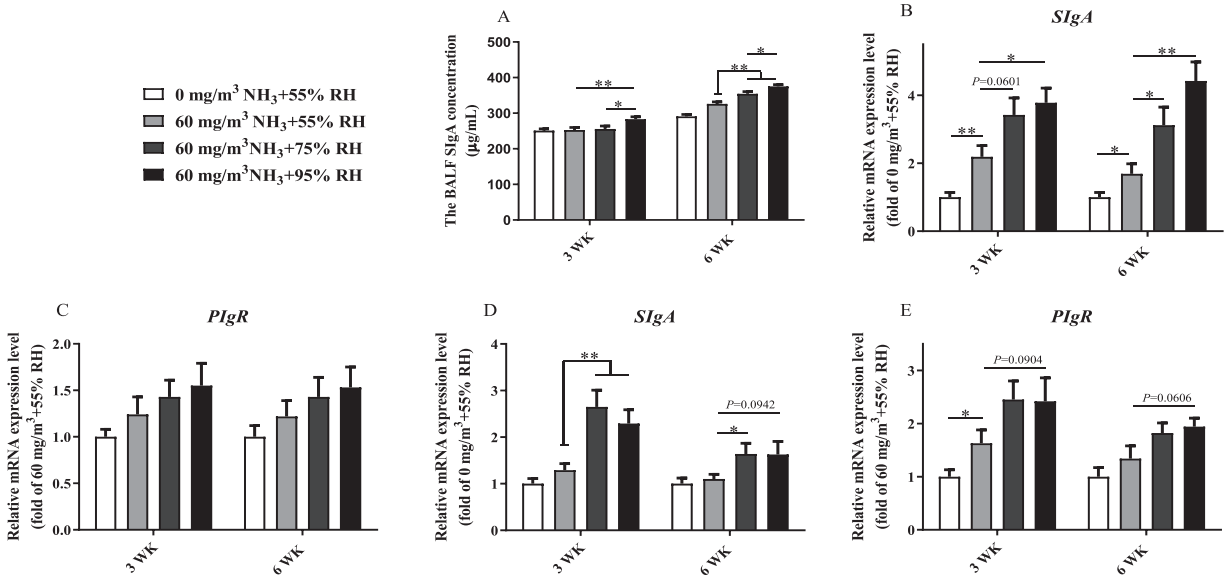

Figure 5. The effect of NH_3 and humidity on the content of SIgA and the gene expression levels of *SIgA* and *PIgR*. A shows the content of SIgA in BALF. (B) and (C) show the genes expression levels of *SIgA* and *PIgR* in the tracheal. (D) and (E) show the genes expression levels of *SIgA* and *PIgR* in the lung. The test data is expressed by the mean ± standard error. * indicates $P < 0.05$; ** indicates $P < 0.01$; n = 8.

NH_3 + 55% RH group ($P < 0.05$, Figures 5A and 5B). Compared to the 60 mg/m^3 NH_3 + 55% RH group, the combination of 60 mg/m^3 NH_3 with 75 or 95% relative humidity significantly increased the content and gene expression level of *SIgA* in lung and BALF ($P < 0.05$, Figures 5A and 5B) at 3rd and 6th wk, respectively.

The Gene Expressions of Cytokines in the Trachea and Lung

The imbalance of Th1/Th2 was assessed by measuring the mRNA levels of *IFN-γ, IL-2, IL-4*, and *IL-10* in the trachea and lung (Figure 6). Compared with the 0 mg/m^3 NH_3 + 55% RH group, the laying hens in the 60 mg/m^3 NH_3 + 55% RH group showed upregulated expression of *IL-1β, IL-4*, and *IL-10* in the trachea ($P < 0.05$, Figures 6B, 6C and 6F) at 3rd wk. At the same time, the 60 mg/m^3 NH_3 + 75% RH significantly inhibited mRNA level of *IFN-γ* ($P < 0.05$, Figure 6C). The 60 mg/m^3 NH_3 + 95% RH remarkably increased the mRNA level of *IL-1β* ($P < 0.05$, Figure 6B) but inhibited the mRNA level of *IFN-γ* and *IL-2* in the trachea ($P < 0.05$, Figures 6C and 6D) at 3rd wk. In the 6th wk, the 60 mg/m^3 NH_3 + 55% RH enhanced the gene expression levels of *TNF-α, IL-1β, IL-4*, and *IL-10* ($P < 0.05$, Figures 6A, 6B, 6E and 6F), and but reduced the gene expression levels of *IFN-γ* expression in the trachea ($P < 0.05$, Figure 6C). Compared with the 60 mg/m^3 NH_3 + 55% RH group, laying hens in the 60 mg/m^3 NH_3 + 95% RH group showed obviously increased gene expression levels of *TNF-α* and *IL-4* ($P < 0.05$, Figures 6A and 6E), and but inhibited gene expression level of *IFN-γ* expression was downregulated in the trachea ($P < 0.05$, Figure 6C) at 6th wk.

The lung tissues exhibited similar phenotypes to the tracheal results. At the 3rd wk, laying hens in 60 mg/m^3 NH_3 + 55% RH group showed significant increase in the gene expression level of *IL-4* ($P < 0.05$, Figure 6K) but reduced gene expression of *IFN-γ* in lung tissues ($P < 0.05$, Figure 6I) compared to 0 mg/m^3 NH_3 + 55% RH group. Meanwhile, 60 mg/m^3 NH_3 + 95% RH obviously decreased the mRNA level of *IL-2* than 60 mg/m^3 NH_3 + 55% RH and 60 mg/m^3 NH_3 + 75% RH group ($P < 0.05$, Figure 6J) at the 3rd wk. At the 6th wk, stimulation of 60 mg/m^3 NH_3 + 55% RH remarkably increased the gene expression level of *IL-4* ($P < 0.05$, Figure 6K) and reduced the gene expression level of *IFN-γ* and *IL-2* in lung tissues compared to 0 mg/m^3 NH_3 + 55% RH group ($P < 0.05$, Figures 6I and 6J). Relative to 60 mg/m^3 NH_3 + 55% RH group, the mRNA level of *IL-1β* was significantly increased in laying hens treated with 60 mg/m^3 NH_3 + 95% RH group ($P < 0.05$, Figure 6H).

The Genes Expression of NF-κB Signaling Pathway in the Trachea and Lungs

The NF-κB signaling pathway is involved in the proinflammatory response and contributes to the production of proinflammatory cytokines. More so, the NF-κB signaling pathway can be activated by the proinflammatory cytokines such as TNF-α and IL-1β. To further verify whether the combination of NH_3 and humidity activates the NF-κB signaling, we detected the mRNA levels of genes related to the NF-κB signaling pathway (Figure 7). Under the 55% relative humidity, exposure to 60 mg/m^3 NH_3 significantly upregulated the gene expression level of *NF-κB, COX2*, and *iNOS* at the 3rd and 6th wk ($P < 0.05$, Figures 7A, 7D and 7E), and significantly upregulated the gene expression level of *I-KK-β* in the trachea at the 6th wk ($P < 0.05$, Figure 7C).

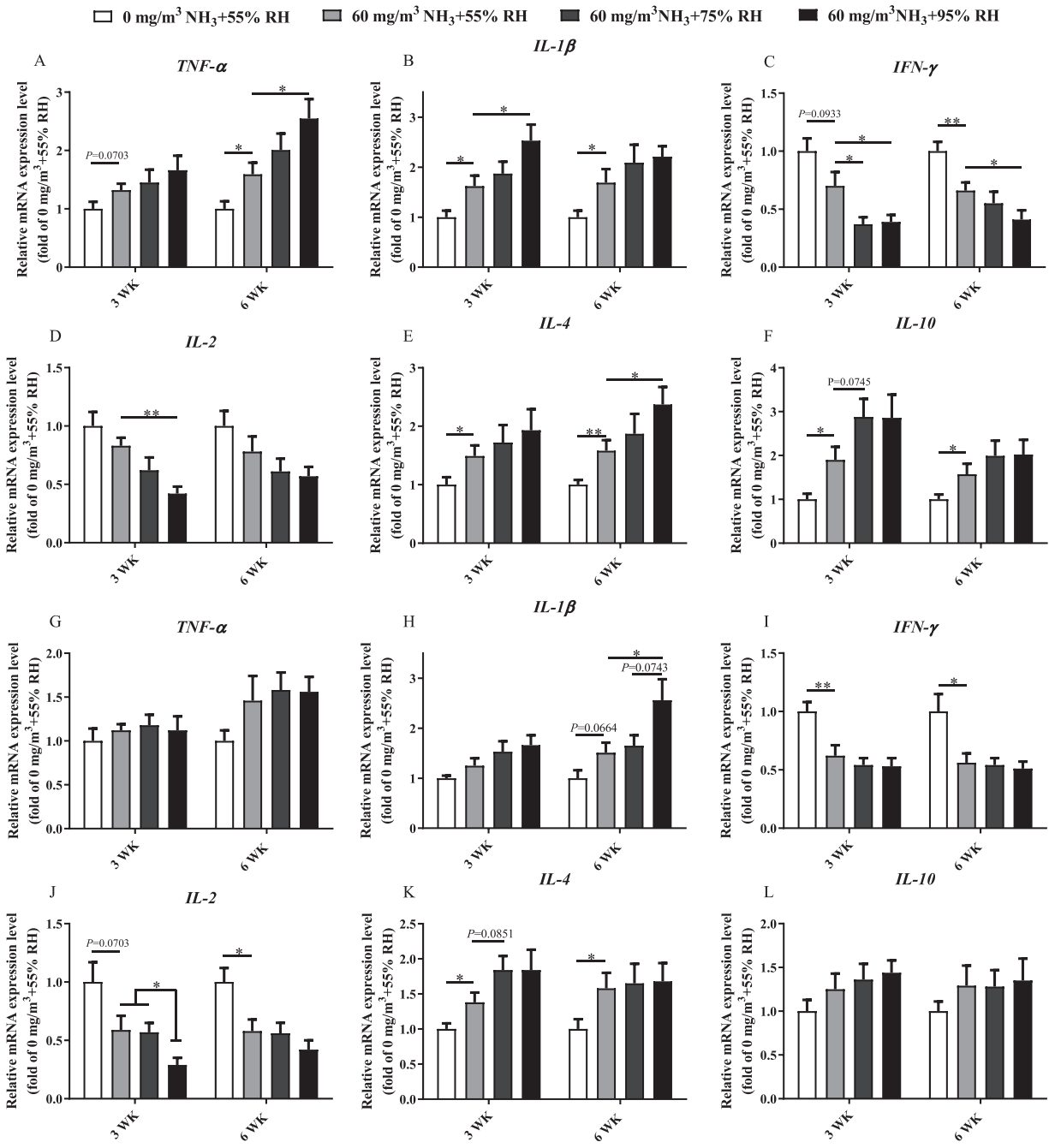

Figure 6. The effect of NH_3 and humidity on the mRNA expression of inflammatory factors. (A)−(F) show the genes expression levels of inflammatory factors in the tracheal. (G)−(L) show the genes expression levels of inflammatory factors in the lung. * indicates $P < 0.05$; ** indicates $P < 0.01$; n = 8.

Compared with the 60 mg/m^3 NH_3 + 55% RH group, the gene expression levels of *NF-κB* and *iNOS* were higher in the trachea of laying hens in the 60 mg/m^3 NH_3 + 95% RH group at the 6th wk ($P < 0.05$, Figures 7A and 7E).

In the lungs, the mRNA levels of *I-κB-α* and *iNOS* in the 60 mg/m^3 NH_3 + 55% RH group were higher than those in the 0 mg/m^3 NH_3 + 55% RH group at the 3rd wk ($P < 0.05$, Figures 7G and 7J). Similar to the trachea results, the mRNA levels of *NF-κB, I-KK-β*, and *COX2* in the 60 mg/m^3 NH_3 + 55% RH group were significantly upregulated in the lungs compared to the 0 mg/m^3 NH_3 + 55% RH group at the 6th wk ($P < 0.05$, Figures 7F, 7H and 7I). Compared with 60 mg/m^3 NH_3 + 55% RH group, the laying hens in the 60 mg/m^3 NH_3 + 95% RH group showed significantly increased gene expression level of *NF-κB* ($P < 0.05$, Figure 7F).

DISCUSSION

Ammonia is the most common harmful pollutant in livestock and poultry production (Wang et al., 2020). After being inhaled by animals, a high concentration of NH_3 directly enters the blood through the alveolar epithelium. NH_3 passing through the blood circulation can

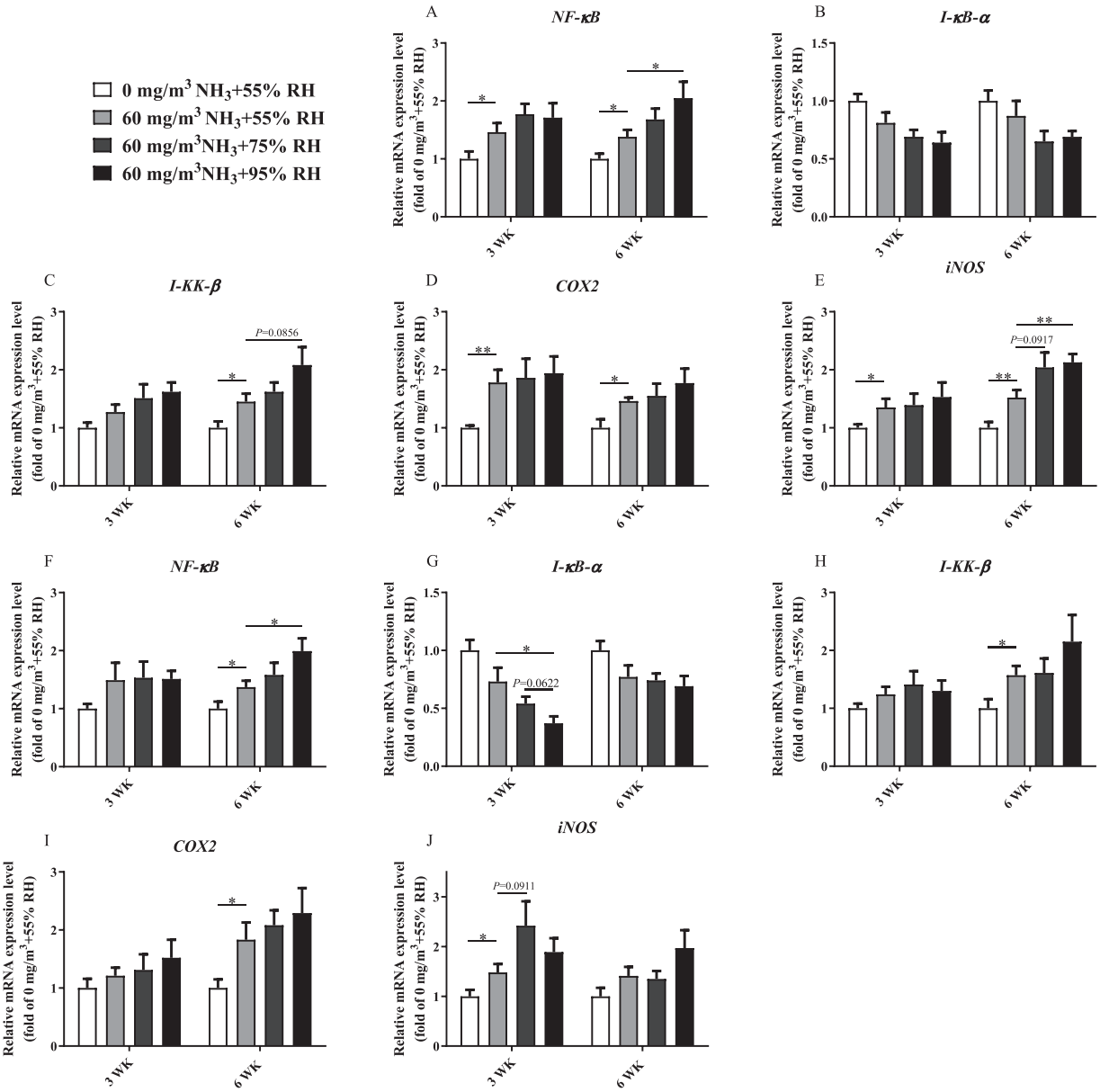

Figure 7. The effect of NH_3 and humidity on the mRNA expression of NF-κB signaling pathway-related genes in lung and trachea of laying hens. (A)−(E) show the genes expression levels in the tracheal. (F)−(J) show the genes expression levels in the lung. The test data is expressed by the mean ± standard error. * indicates $P < 0.05$; ** indicates $P < 0.01$; n = 8.

be converted into uric acid in the liver and kidneys and is then excreted through the feces. However, most of the ammonia remains in the blood. The present research found that NH_3 exposure significantly increased the content of blood ammonia. Importantly, the content of blood ammonia increased significantly with an increase in relative humidity. We suggest that in a highly humid environment, more NH_3 may be dissolved in the air droplets and inhaled into the blood during respiration by laying hens, consequently increasing the blood ammonia content. Although the liver has limited ability to convert ammonia, more inhaled NH_3 still leads to elevated blood ammonia levels.

NH_3 in highly humid air was dissolved into the mucosa of the respiratory tract, elevating the mucosal pH. An increase in NH_3 content and mucosal pH can weaken the clearance ability and cause loss of cilia, which increases the prevalence of tracheitis and bacterial diseases in poultry (David and Richard, 2011; Shi et al., 2019; Liu et al., 2020). Research showed that when broilers were exposed to 70 mg/m^3 NH_3, parts of the tracheal cilia were severely damaged and shed (Beker et al., 2004). This study found that 60 mg/m^3 NH_3 damaged the tissue structure of the tracheal cilia, and the blood vessels of the lungs were swollen and hemorrhagic in laying hens. Along with the increased blood ammonia, the damage was anabatic with the increase in relative humidity during the 60 mg/m^3 NH_3 exposure. In the 60 mg/m^3 NH_3 + 75% RH or 95% RH group, the deciduous range of tracheal cilia was increased, and the pulmonary blood vessels appeared congested, and edematous. In addition, the infiltration of inflammatory cells was increased in the tracheal and lung mucosa. Therefore, these morphological characteristics suggest that increased humidity can aggravate NH_3-induced damage to the respiratory system, resulting in tracheitis and pneumonia.

Maintaining the integrity of tight junctions is essential for the functioning of the mechanical barrier of the respiratory tract, in which the Occludin, Claudin, and ZO play the major effects (Tatsuta et al., 2019). According to the downregulated mRNA levels of tight junction proteins in the trachea and lung, exposure to 60 mg/m^3 NH_3 resulted in a damaged mechanical barrier of the respiratory tract. However, the combination of 60 mg/m^3 NH_3 and 95% relative humidity led to lowered Occludin, Claudin-1, and ZO-1 expressions in the trachea and lung of laying hens. This indicates that an increase in humidity aggravates the damage to the mechanical barrier of the respiratory tract. Mucin is an important component of respiratory mucus. Generally, the secretion of mucin increases when the respiratory tract is stimulated by harmful substances (Linden et al., 2008). When the mucus secretion was increased, the goblet cells of the respiratory tract were hypertrophic and proliferative, and after being blocked by increased mucus, the local defense capability of the respiratory tract was weakened (Rose et al., 2001). In this study, exposure to NH_3 induced mucin secretion in the respiratory tract, along with an increased MUC5AC expression. These results indicate that NH_3 dissolved in the respiratory mucus stimulated the secretion of mucin and altered the homeostasis of the mucus layer in the respiratory tract. With an increase in humidity, the mucin content in BALF and the gene expression of MUC5AC were significantly upregulated, indicating that high humidity in poultry houses aggravates NH_3 damage to the chemical barrier of the respiratory tract in laying hens. As the first line of immune defense, the SIgA exists in the mucus of the respiratory tract and protects against pathogen invasion (Jenny and Michael, 2006). SIgA can neutralize pathogens by forming complexes, which are removed by the cilia and mucus during immune clearance (Bonner et al., 2008). Similar to the mucin, NH_3 stimulated the synthesis and secretion of SIgA in BALF. The increase in relative humidity aggravated the synthesis and secretion of SIgA. Cytokines secreted by Th2 cells, such as TGF-β and IL-4, can promote the secretion of SIgA. It was reported that IL-4 promoted the production of SIgA-positive B cells (Warner et al., 1999). In this study, NH_3 exposure increased the expression of IL-4. Therefore, the elevation in IL-4 may act to enhance SIgA synthesis in the respiratory mucosa, thus contributing to resistance against pathogen invasion. Therefore, the increase in humidity aggravated the damage induced by NH_3 exposure on the respiratory tract barrier.

External stress factors including harmful air pollutants can affect the function of immune cells and secreted cytokines (Glencross et al., 2020). The balance of Th1/Th2 is an important mechanism in maintaining the immune function of the body. A disordered Th1/Th2 leads to an inflammatory response in the lungs of chicken (An et al., 2019; Zhao et al., 2020). More so, the imbalance of Th1/Th2 can activate the NF-κB pathway in the respiratory tract (Chang et al., 2017). Th1 cells mainly secrete IFN-γ and IL-2 cytokines. A previous study had reported that the inhibition of IFN-γ expression was related to suppressed Th1 immune function (Crusz and Balkwill, 2015). However, Th2 cells mainly secrete IL-4 and IL-10. In the respiratory tract, downregulation of IFN-γ induced by SO_2 in asthmatic mice caused respiratory inflammation (Li et al., 2014), which corroborated the findings of this study. After inhaling 60 mg/m^3 NH_3, the decreased expressions of IFN-γ and IL-2 indicated that the immune function of Th1 cells was inhibited by NH_3. The increased expressions of IL-4 and IL-10 indicated that NH_3 stimulation enhances the immune response of Th2 cells. The increased expression of IFN-γ and IL-2 and reduced expression of IL-4 and IL-10 showed that NH_3 induced an imbalance of Th1/Th2 in the respiratory mucosa, resulting in immune dysregulation. However, an increased humidity aggravated the degree of Th1/Th2 imbalance induced by NH_3. According to the tissue sections and imbalance of Th1/Th2, it can be inferred that exposure to NH_3 caused tracheitis and pneumonia in laying hens. As major proinflammatory factors, TNF-α and IL-1β have multiple roles in the regulation of inflammation (Crusz and Balkwill, 2015; Sun et al., 2017). TNF-α and IL-1β are related to inflammation caused by the activation of the NF-κB pathway and they also activate the NF-κB pathway (Goebeler et al., 2001; Baker et al., 2011; Liu et al., 2017). Previous research had found that excessive NH_3 upregulated IL-1β expression in the spleen of laying hens, leading to splenic inflammation (Wu et al., 2017; Zhao et al., 2020). Our results showed that NH_3 exposure led to the upregulation of TNF-α and IL-1β expression in the trachea and lung, and the combined treatment of NH_3 with increased humidity further modulated their expressions. These results indicate that the NF-κB pathway may be activated by either NH_3 or the combination of NH_3 and increased humidity. NF-κB signaling pathway plays an extremely important role in the development of NH_3-induced inflammation, via regulating the expression levels of cytokines in the immune system, leading to tissue damage (An et al., 2019; Shi et al., 2019; Chen et al., 2020). Therefore, we detected the expression levels of genes related to the NF-κB signaling pathway. Results showed that the gene expression levels of I-KK-β and NF-κB and their downstream targets, COX2 and iNOS were upregulated. On the contrary, the expression level of I-κB-α was downregulated. This suggests that the NF-κB signaling pathway was activated by both NH_3 and increased humidity.

In conclusion, this study demonstrated that NH_3 stimulation damaged the respiratory mucosal barrier and induced respiratory mucosal inflammation and that this condition was aggravated under high humidity in laying hens. NH_3 induced an imbalance of Th1/Th2 and activated the NF-κB signaling pathway, which triggered inflammation of the respiratory tract (Figure 8). Also, the combination of NH_3 and high humidity caused severe inflammatory damage to the respiratory tract of laying hens via a similar route but with stronger effects.

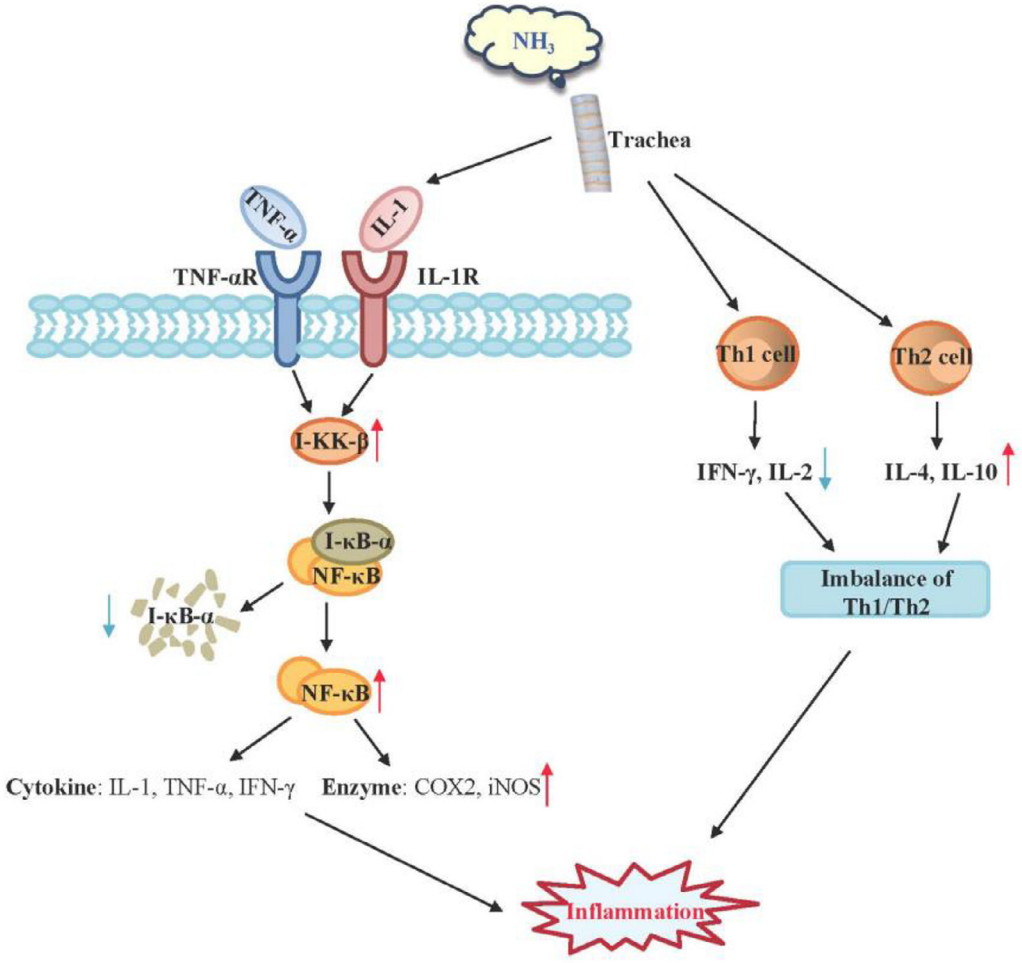

Figure 8. Ammonia induces imbalance of Th1/Th2 cells and activation of NF-κB signaling pathway leading to respiratory tract inflammation.

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DISCLOSURES

The authors declare no competing interests.

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