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 (NH3) 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 NH3 on the respiratory status of birds, causing severe physiological consequences. In this study, the combined effects of NH3 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 NH3 alone or NH3 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 NH3 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, cyclooxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS) were significantly increased. In conclusion, NH3 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 NH3 in poultry.

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

INTRODUCTION

Ammonia (NH3) is a harmful gas and an important source of air pollution. Within the environment, NH3 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 NH3. 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 NH3 and hydrogen sulfide (Louis et al., 2014). Under the effect of urease, the uric acid excreted by the kidneys is also decomposed into NH3 gas and emitted into the environment (Davila et al., 2013). High levels of NH3 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. NH3 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 NH3 suffer an increased infiltration of inflammatory substances into
shown that NH3 stimulated changes in the cytokines (Peterson et al., 2007; Cerutti, 2008). Studies have suggested that NH3 induces cytokine secretion (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 NH3 stimulated changes in the cytokines secreted by Th2 and Th17, which in turn aggravated the imbalance of Threg/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 (SO2) induced asthma in rats, and the hydrogen sulfide (H2S) 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 NH3 (Groot Koerkamp and Bleijenberg, 1998). It was reported that the concentration of NH3 in the poultry house increased with an increase in humidity, which aggravated the adverse effects of NH3 on the production performance of livestock and poultry (Weaver and Meijerhof, 1991).

This study examined the detrimental effects of NH3 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 NH3 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/m3 NH3 + 55% RH, 60 mg/m3 NH3 + 55% RH, 60 mg/m3 NH3 + 75% RH, and 60 mg/m3 NH3 + 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 for each 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
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).

**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              |
| Claudin-1 | NM_001013611.2               | ACAAATGGTGGGCATCAG         | Reverse     | 182              |
| Occludin | XM_046904540.1               | GCTTTCCTTCACCCCTGTCCTCA   | Forward     | 154              |
| ZO-1   | XM_046925214.1            | GCGGCTTGTTCTCAGGCTGCTC   | Reverse     | 131              |
| MUC2   | XM_040673077.2            | TGACAGCCATCAAGGACACA     | Forward     | 143              |
| MUC5AC | XM_040673078.2            | AGAGGCGCAATTTATTTGCTACAC  | Reverse     | 244              |
| S IgA  | S04061.1                | AGGGAAGCTGGTGCTGCAAG      | Forward     | 114              |
| PlgR   | NM_001044644.2            | GCCCTTCTGCATACAGTGAAGGAC  | Forward     | 145              |
| TNF-α  | XM_04927265.1            | GGGGCGGAACAGGCAGAAAT      | Reverse     | 123              |
| IL-1β  | XM_046931582.1            | TCTTCTACGCGCTGGGACACG     | Forward     | 111              |
| IFN-γ  | NM_205149.2              | TAGGTGGGAGTGTTGACCTG     | Reverse     | 80               |
| IL-2   | NM_204153.2              | GACAGCAGCCCAAGTCAAGGGAG  | Forward     | 102              |
| IL-4   | NM_001004144.4            | TGATAGGCTGGAGGGAGGAGG     | Reverse     | 162              |
| IL-10  | NM_001000144.4            | TCGGTCGAGTTCAGGCTGCTC    | Reverse     | 106              |
| NF-κB  | XM_046915553.1            | TCGGTCGAGTTCAGGCTGCTC    | Reverse     | 106              |
| i-KK-β | XM_046931637.1            | TGGATAGGCTGGAGGGAGGAGG    | Reverse     | 92               |
| i-KB-α | NM_001001472.3            | TCGGTCGAGTTCAGGCTGCTC    | Reverse     | 84               |
| COX2   | XM_046922435.1            | TCGGTCGAGTTCAGGCTGCTC    | Reverse     | 82               |
| iNOS   | NM_204961.2              | TCGGTCGAGTTCAGGCTGCTC    | Reverse     | 82               |

**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 (Mibio 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 $2^{-\Delta\Delta Ct}$ 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/m³ NH₃ + 55% RH and 60 mg/m³ NH₃ + 55% RH) and the effect of humidity at the same ammonia concentration (60 mg/m³ NH₃ + 55% RH, 60 mg/m³ NH₃ + 75% RH, and 60 mg/m³ NH₃ + 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 NH₃ 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/m³ NH₃ group increased significantly in the 3rd wk (\( P < 0.05 \), Figure 1) and 6th wk (\( P < 0.01 \), Figure 1) compared to 0 mg/m³ NH₃ group. When the concentration of ammonia was 60 mg/m³, the blood ammonia content in 60 mg/m³ + 95% RH group was significantly higher than that in 60 mg/m³ + 55% 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/m³ + 55% RH group.

**The Effect of NH₃ 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/m³ NH₃ + 55% RH group was normal. The tracheal cilia were arranged neatly and there was no adhesion. Relatively, the stimulation of 60 mg/m³ NH₃ resulted in the fall, loss, and adhesion of tracheal cilia in the 60 mg/m³ NH₃ + 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/m³ NH₃ + 95% RH group showed increased infiltration of inflammatory cells and interstitial space (Figures 2A−2D).

**The Effect of NH₃ 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/m³ NH₃ significantly downregulated the expression of ZO-1 in the trachea and Occludin in the lungs in the 3rd wk compared to the 0 mg/m³ NH₃ + 55% RH group (\( P < 0.05 \), Figures 3C and 3E). At the 6th wk, 60 mg/m³ NH₃ significantly downregulated the gene expression levels of Occludin and ZO-1 in both trachea and lung tissues compared to the 0 mg/m³ NH₃ + 55% RH group (\( P < 0.05 \), Figures 3B, 3C, 3E and 3F). At the 60 mg/m³ NH₃ concentration, relative to 60 mg/m³ NH₃ + 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/m³ NH₃ + 75% RH significantly decreased the gene expression level of ZO-1 compared to 60 mg/m³ NH₃ + 55% RH group (\( P < 0.05 \), Figure 3C). Importantly, 60 mg/m³ NH₃ + 95% RH significantly

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**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.
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 0 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).
The Effect of NH₃ 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/m³ NH₃ 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/m³ NH₃, 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/m³ NH₃ + 95% relative humidity showed higher SIgA content and gene expression level in the BALF than those in the 60 mg/m³ NH₃ + 55% RH group (P < 0.01) at the 3rd and 6th wk.

**Figure 3.** The effect of NH₃ 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.

**Figure 4.** The effect of NH₃ 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.
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-$\gamma$, 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-1$\beta$, 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-$\gamma$ ($P < 0.05$, Figure 6C). The 60 mg/m$^3$ NH$_3$ + 95% RH remarkably increased the mRNA level of IL-1$\beta$ 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-$\gamma$ 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-1$\beta$ 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-$\kappa$B Signaling Pathway in the Trachea and Lungs

The NF-$\kappa$B signaling pathway is involved in the proinflammatory response and contributes to the production of proinflammatory cytokines. More so, the NF-$\kappa$B signaling pathway can be activated by the proinflammatory cytokines such as TNF-$\alpha$ and IL-1$\beta$. To further verify whether the combination of NH$_3$ and humidity activates the NF-$\kappa$B signaling, we detected the mRNA levels of genes related to the NF-$\kappa$B 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-$\kappa$B, 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-$KK$-$\beta$ in the trachea at the 6th wk ($P < 0.05$, Figure 7C).
Compared with the 60 mg/m³ NH₃ + 55% RH group, the gene expression levels of NF-κB and iNOS were higher in the trachea of laying hens in the 60 mg/m³ NH₃ + 95% RH group at the 6th wk (P < 0.05, Figures 7A and 7E). In the lungs, the mRNA levels of IκB-α and iNOS in the 60 mg/m³ NH₃ + 55% RH group were higher than those in the 0 mg/m³ NH₃ + 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, IκB-β, and COX2 in the 60 mg/m³ NH₃ + 55% RH group were significantly upregulated in the lungs compared to the 0 mg/m³ NH₃ + 55% RH group at the 6th wk (P < 0.05, Figures 7F, 7H and 7I). Compared with 60 mg/m³ NH₃ + 55% RH group, the laying hens in the 60 mg/m³ NH₃ + 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 NH₃ directly enters the blood through the alveolar epithelium. NH₃ passing through the blood circulation can...
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 NH₃ 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 NH₃ 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 NH₃ still leads to elevated blood ammonia levels.

NH₃ in highly humid air was dissolved into the mucosa of the respiratory tract, elevating the mucosal pH. An increase in NH₃ 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/m³ NH₃, parts of the tracheal cilia were severely damaged and shed (Beker et al., 2004). This study found that 60 mg/m³ NH₃ 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/m³ NH₃ exposure. In the 60 mg/m³ NH₃ + 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 NH₃-induced damage to the respiratory system, resulting in tracheitis and pneumonia.

Figure 7. The effect of NH₃ 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.
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/m³ NH₃ resulted in a damaged mechanical barrier of the respiratory tract. However, the combination of 60 mg/m³ NH₃ 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 mucous 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 NH₃ induced mucin secretion in the respiratory tract, along with an increased MUC5AC expression. These results indicate that NH₃ 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 NH₃ 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, NH₃ 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, NH₃ 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 NH₃ 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 SO₂ in asthmatic mice caused respiratory inflammation (Li et al., 2014), which corroborated the findings of this study. After inhaling 60 mg/m³ NH₃, the decreased expressions of IFN-γ and IL-2 indicated that the immune function of Th1 cells was inhibited by NH₃. The increased expressions of IL-4 and IL-10 indicated that NH₃ 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 NH₃ 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 NH₃. According to the tissue sections and imbalance of Th1/Th2, it can be inferred that exposure to NH₃ 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 NH₃ 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 NH₃ exposure led to the upregulation of TNF-α and IL-1β expression in the trachea and lung, and the combined treatment of NH₃ with increased humidity further modulated their expressions. These results indicate that the NF-κB pathway may be activated by either NH₃ or the combination of NH₃ and increased humidity. NF-κB signaling pathway plays an extremely important role in the development of NH₃-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 NH₃ and increased humidity.

In conclusion, this study demonstrated that NH₃ stimulation damaged the respiratory mucosal barrier and induced respiratory mucosal inflammation and that this condition was aggravated under high humidity in laying hens. NH₃ 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 NH₃ and high humidity caused severe inflammatory damage to the respiratory tract of laying hens via a similar route but with stronger effects.
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DISCLOSURES

The authors declare no competing interests.

REFERENCES

An, Y., H. Xing, Y. Zhang, P. Jia, X. Gu, and X. Teng. 2019. The evaluation of potential immunotoxicity induced by environmental pollutant ammonia in broilers. Poult. Sci. 98:3165–3175.

Baker, R. G., M. S. Hayden, and S. Ghosh. 2011. NF-κB, inflammation, and metabolic disease. Cell Metab. 13:11–22.

Beker, A., S. L. Vanooser, J. H. Swartzlander, and R. G. Teeter. 2004. Atmospheric ammonia concentration effects on broiler growth and performance. J. Appl. Poult. Res. 13:5–9.

Beusen, A. H. W., A. F. Bouwman, P. S. C. Heuberger, G. V. Drecht, and K. W. V. D. Hock. 2008. Bottom-up uncertainty estimates of global ammonia emissions from global agricultural production systems. Atmos. Environ. 42:6067–6077.

Bonner, A., P. B. Furtado, A. Almogren, M. A. Kerr, and S. J. Perkins. 2008. Implications of the near-planar solution structure of human myeloma dimeric IgA1 for mucosal immunity and IgA nephropathy. J. Immunol. 180:1008–1018.

Cerutti, A. 2008. The regulation of IgA class switching. Nat. Rev. Immunol. 8:421–434.

Chang, X., A. Zhu, F. Liu, L. Zou, L. Su, S. Li, and Y. Sun. 2017. Role of NF-κB activation and Th1/Th2 imbalance in pulmonary toxicity induced by nano NiO. Environ. Toxicol. 32:1354–1362.

Chen, D., F. Ning, J. Zhang, Y. Tang, and X. Teng. 2020. NF-κB pathway took part in the development of apoptosis mediated by miR-15a and oxidative stress via mitochondrial pathway in ammonia-treated chicken splenic lymphocytes. Sci. Total Environ. 729:139017.

Cruz, S. M., and F. R. Balkwill. 2015. Inflammation and cancer: advances and new agents. Nat. Rev. Oncol. 12:584–596.

Curtis, S. E., C. R. Anderson, J. Simon, A. H. Jensen, D. L. Day, and K. W. Kelley. 1975. Effects of aerial ammonia, hydrogen sulfide and swine-house dust on rate of gain and respiratory-tract structure in swine. J. Anim. Sci. 41:735–739.

David, P., and L. Richard. 2011. Epithelial cells and airway diseases. Immunol. Rev. 242:186–204.

Davila, A. M., F. Blachier, M. Gotteland, M. Andriamihaja, P. H. Benetti, Y. Sanz, and D. Tomé. 2013. Intestinal luminal nitrogen metabolism: role of the gut microbiota and consequences for the host. Pharmacol. Res. 69:114–126.

Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Robers, and F. Smith. 1956. Colorimetric method for determination of sugar and related substances. Anal. Chem. 28:350–356.
Glencross, D. A., T. R. Ho, N. Camiña, C. M. Hawrylowlcz, and P. E. Pfeffer. 2020. Air pollution and its effects on the immune system. Free Radic. Biol. Med. 151:56–68.

Goebeler, M., R. Gillitzer, K. Kilian, K. Utzel, E. B. Bröcker, U. R. Rapp, and S. Ludwig. 2001. Multiple signaling pathways regulate NF-κB-dependent transcription of the monocyte chemotactic protein-1 gene in primary endothelial cells. Blood 97:46–55.

Greene, C. M., N. J. Hutchings, H. D. Haenel, B. Amon, H. Menzi, M. H. Mikkelsen, T. H. Misselbrook, C. van Bruggen, T. Kupper, and J. Webb. 2019. Comparison of ammonia emissions related to nutrient use efficiency of livestock production in Europe. J. Clean Prod. 211:1162–1170.

Groot Koerkamp, P. W., and R. Bleijenberg. 1998. Effect of type of aviary, manure and litter handling on the emission kinetics of ammonia from layer houses. Br. Poult. Sci. 39:379–392.

Jenny, M. W., and A. K. Michael. 2006. The function of immunoglobulin A in immunity. J. Pathol. 208:270–282.

Johnson, D. C. 2011. Airway mucus function and dysfunction. N. Engl. J. Med. 364:2233–2247.

Li, R., X. Kou, J. Tian, Z. Meng, Z. Cai, F. Cheng, and C. Dong. 2014. Effect of sulfur dioxide on inflammatory and immune regulation in asthmatic rats. Chemosphere 112:296–304.

Linden, S. K., P. Sutton, N. G. Karlsson, V. Korolik, and M. A. McGuckin. 2008. Mucins in the mucosal barrier to infection. Mucosal. Immunol. 1:183–197.

Liu, Q. X., Y. Zhou, X. M. Li, D. D. Ma, S. Xing, J. H. Feng, and M. H. Zhang. 2020. Ammonia induce lung tissue injury in broilers by activating NLRP3 inflammasome via Escherichia/Shigella. Poult. Sci. 99:3402–3410.

Liu, T., L. Zhang, D. Joo, and S. C. Sun. 2017. NF-κB signaling in inflammation. Signal Transduct. Target Ther. 2:1–9.

Louis, P., G. L. Hold, and H. J. Flint. 2014. The gut microbiota, bacterial metabolites and colorectal cancer. Nat. Rev. Microbiol. 12:661–672.

Luan, Z. G., H. Zhang, P. T. Yang, X. C. Ma, C. Zhang, and R. X. Guo. 2010. HMGB1 activates nuclear factor-κB signaling by RAGE and increases the production of TNF-α in human umbilical vein endothelial cells. Immunobiology 215:956–962.

Macpherson, A. J., L. Hunziker, K. McCoy, and A. Larramé. 2001. IgA responses in the intestinal mucosa against pathogenic and non-pathogenic microorganisms. Microbes Infect. 3:1021–1035.

Miles, D. M., W. W. Miller, S. L. Branton, W. R. Maslin, and B. D. Lott. 2006. Ocular responses to ammonia in broiler chickens. Avian Dis 50:45–49.

Naseem, S., and A. J. King. 2018. Ammonia production in poultry houses can affect health of humans, birds, and the environment—techniques for its reduction during poultry production. Environ. Sci. Pollut. Res. Int. 25:15269–15293.

Peterson, D. A., N. P. McNulty, J. L. Guruge, and J. I. Gordon. 2007. IgA Response to symbiotic bacteria as a mediator of gut homeostasis. Cell Host Microbe 2:328–339.

Shi, Q., W. Wang, M. Chen, H. Zhang, and S. Xu. 2019. Ammonia induces Treg/Th1 imbalance with triggered NF-κB pathway leading to chicken respiratory inflammation response. Sci. Total Environ. 659:354–362.

Sun, Z., C. Liu, T. Pan, H. Yao, and S. Li. 2017. Selenium accelerates chicken dendritic cells differentiation and affects selenoproteins expression. Dev. Comp. Immunol. 12:30–37.

Sutton, M. A., R. Stefan, S. N. Riddick, D. Ulrike, N. Eiko, and M. R. Theobald. 2013. Towards a climate-dependent paradigm of ammonia emission and deposition. Philos. Trans. R Soc. Lond. B Biol. Sci. 368:1–13.

Tatsuta, M., K. Kan-O, Y. Ishii, N. Yamamoto, T. Ogawa, S. Fukuyama, A. Ogawa, A. Fujita, Y. Nakashima, and K. Matsumoto. 2019. Effects of cigarette smoke on barrier function and tight junction proteins in the bronchial epithelium: protective role of cathelicidin LL-37. Respir. Res. 20:251.

Thornton, D. J., K. Rousseau, and M. A. Mégucón. 2008. Structure and function of the polymeric mucins in airways mucus. Annu. Rev. Physiol. 70:459–486.

Wang, W., M. Chen, X. Jin, X. Li, Z. Yang, H. Lin, and S. Xu. 2018. H2S induces Th1/Th2 imbalance with triggered NF-κB pathway to exacerbate LPS-induce chicken pneumonia response. Chemosphere 208:241–246.

Wang, W., Q. Shi, S. Wang, H. Zhang, and S. Xu. 2020. Ammonia regulates chicken tracheal cell necroptosis via the LncRNA-1070532933/MiR-148a-3p/FAF1 axis. J. Hazard Mater. 386:121626.

Warner, R. H., F. M. Stevens, and C. F. McCarthy. 1999. Salivary SlgA and SlgA 1 in coeliac disease, inflammatory bowel disease and controls. Ir. J. Med. Sci. 168:33–35.

Weaver, W. D., and R. Meijerhof. 1991. The effect of different levels of relative humidity and air movement on litter conditions, ammonia levels, growth, and carcass quality for broiler chickens. Poult. Sci. 70:746–755.

Wei, F. X., X. F. Hu, R. N. Sa, F. Z. Liu, S. Y. Li, and Q. Y. Sun. 2014. Antioxidant capacity and meat quality of broilers exposed to different ambient humidity and ammonia concentrations. Genet. Mol. Res. 13:3117–3127.

Wu, Y. N., F. F. Yan, J. Y. Hu, H. Chen, C. M. Tucker, A. R. Green, and H. W. Cheng. 2017. The effect of chronic ammonia exposure on acute-phase proteins, immunoglobulin, and cytokines in laying hens. Poult. Sci. 96:1524–1530.

Yahav, S., S. Goldfeld, I. Plavnik, and S. Hurwitz. 1995. Physiological responses of chickens and turkeys to relative humidity during exposure to high ambient temperature. J. Therm. Biol. 20:245–253.

Yahav, S., I. Plavnik, M. Rusal, and S. Hurwitz. 1998. Response of turkeys to relative humidity at high ambient temperature. Br. Poult. Sci. 39:340–345.

Zhang, J., C. Li, X. Tang, Q. Lu, R. Sa, and H. Zhang. 2015. Proteome changes in the small intestinal mucosa of broilers (Gallus galus) induced by high concentrations of atmospheric ammonia. Proteome Sci. 13:9.

Zhao, F., J. Qu, W. Wang, S. Li, and S. Xu. 2020. The imbalance of Th1/Th2 triggers an inflammatory response in chicken spleens after ammonia exposure. Poult. Sci. 99:3817–3822.

Zhao, J., Z. Gao, Z. Tian, Y. Xie, F. Xin, R. Jiang, H. Kan, and W. Song. 2013. The biological effects of individual-level PM2.5 exposure on systemic immunity and inflammatory response in traffic policemen. Occup. Environ. Med. 70:426–431.

Zou, W., P. Yin, Y. Shi, N. Jin, Q. Gao, J. Li, and F. Liu. 2018. A novel biological role of α-mangostin via TAK1-NF-κB pathway against inflammatory. Inflammation 42:103–112.