



## **Atmospheric ammonia causes histopathological lesions, cell cycle blockage, and apoptosis of spleen in chickens**

Authors: Chen, Lin, Cai, Tong, Zhao, Cuiyan, Bai, Shiping, Shu, Gang, et al.

Source: Canadian Journal of Animal Science, 102(3) : 448-456

Published By: Canadian Science Publishing

URL: <https://doi.org/10.1139/cjas-2021-0084>

---

BioOne Complete ([complete.BioOne.org](https://complete.BioOne.org)) is a full-text database of 200 subscribed and open-access titles in the biological, ecological, and environmental sciences published by nonprofit societies, associations, museums, institutions, and presses.

Your use of this PDF, the BioOne Complete website, and all posted and associated content indicates your acceptance of BioOne's Terms of Use, available at [www.bioone.org/terms-of-use](http://www.bioone.org/terms-of-use).

Usage of BioOne Complete content is strictly limited to personal, educational, and non - commercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

---

BioOne sees sustainable scholarly publishing as an inherently collaborative enterprise connecting authors, nonprofit publishers, academic institutions, research libraries, and research funders in the common goal of maximizing access to critical research.

# Atmospheric ammonia causes histopathological lesions, cell cycle blockage, and apoptosis of spleen in chickens

Lin Chen<sup>a</sup>, Tong Cai<sup>b</sup>, Cuiyan Zhao<sup>c</sup>, Shiping Bai<sup>d</sup>, Gang Shu<sup>d</sup>, Changlin Wen<sup>e</sup>, Qinkun Xu<sup>c</sup>, and Xi Peng<sup>a</sup>

<sup>a</sup>College of Food and Biological Engineering, Chengdu University, No. 2025, Chengluo Road, Chengdu, Sichuan Province, 610106, China; <sup>b</sup>Sichuan Youngster Technology Co. Ltd., No. 18, Bayi Road, Wenjiang District, Chengdu, Sichuan Province, 611130, China; <sup>c</sup>Henry Fok College of Biology and Agriculture, Shaoguan University, No. 288, University Road, Zhenjiang District, Shaoguan City, Guangdong Province, 512005, China; <sup>d</sup>Animal Nutrition Institute, College of Veterinary Medicine, Sichuan Agricultural University, No. 211, Huiming Road, Wenjiang District, Chengdu, Sichuan Province, 611130, China; <sup>e</sup>Sichuan Industrial Institute of Antibiotics, School of Pharmacy, Chengdu University, No. 2025, Chengluo Road, Chengdu, Sichuan Province, 610106, China

Corresponding authors: Xi Peng (email: pengxi@cdu.edu.cn); Shiping Bai (email: shipingbai@sicau.edu.cn)

## Abstract

This experiment was conducted to investigate the effect of atmospheric ammonia (NH<sub>3</sub>) on histological changes, cell cycle distribution, and apoptosis of the spleen in chickens. Two hundred forty chickens were randomly allocated to the control group (without NH<sub>3</sub> challenge) and NH<sub>3</sub> group (70 ± 5 ppm NH<sub>3</sub>). The experiment lasted for 8 days. The results showed that NH<sub>3</sub> exposure caused decreased relative weight ( $P < 0.05$ ), dysplasia of white pulps, obvious ultrastructural lesions, upregulation of G<sub>0</sub>G<sub>1</sub> phase cells, excessive apoptosis, and oxidative stress ( $P < 0.05$ ) in the spleen. The mechanisms of cell cycle blockage were closely related to the upregulation of the P53 and P21 genes ( $P < 0.05$ ), the downregulation of the cyclin D1 and CDK6 genes ( $P < 0.05$ ), and the decrease of proliferating cell nuclear antigen (PCNA) protein ( $P < 0.05$ ). The activated apoptosis resulted from the increased gene and protein expression of Bax and caspase-3 ( $P < 0.05$ ), and the decreased gene and protein expression of Bcl-2 ( $P < 0.05$ ). The results suggested that 70 ± 5 ppm NH<sub>3</sub> caused the spleen dysplasia, which was closely related to the cell cycle arrest and mitochondrial apoptotic pathway activation.

**Key words:** ammonia, chicken, spleen, histopathology, cell cycle, apoptosis

## Résumé

L'expérience a été effectuée afin d'évaluer les effets de l'ammoniac (NH<sub>3</sub>) atmosphérique sur les changements histologiques, la distribution du cycle cellulaire, et l'apoptose de la rate chez les poulets. 240 poulets ont été alloués de façon aléatoire aux groupes témoin (sans provocation NH<sub>3</sub>) et NH<sub>3</sub> (70 ± 5 ppm NH<sub>3</sub>). L'expérience a duré huit jours. Les résultats ont montré que l'exposition à NH<sub>3</sub> a provoqué une diminution du poids relatif ( $P < 0,05$ ), une dysplasie des pulpes blanches, des lésions ultra-structurales évidentes, une régulation positive des cellules en phase G<sub>0</sub>G<sub>1</sub>, une apoptose excessive, et un stress oxydatif ( $P < 0,05$ ) dans la rate. Les mécanismes de blocage du cycle cellulaire étaient reliés étroitement à la régulation positive des gènes de P53 et P21 ( $P < 0,05$ ), à la régulation négative des gènes de CyclinD 1 et CDK 6 ( $P < 0,05$ ), et à la diminution de la protéine antigène nucléaire de prolifération cellulaire (PCNA — « proliferating cell nuclear antigen ») ( $P < 0,05$ ). L'activation de l'apoptose pourrait être imputable à l'augmentation de l'expression des gènes et protéines Bax et caspase-3 ( $P < 0,05$ ), et la diminution de l'expression du gène et de la protéine Bcl-2 ( $P < 0,05$ ). Les résultats suggèrent que 70 ± 5 ppm NH<sub>3</sub> ont provoqué une dysplasie de la rate, qui étaient étroitement reliés à l'arrêt du cycle cellulaire et l'activation de la voie apoptotique des mitochondries. [Traduit par la Rédaction]

**Mots-clés :** ammoniac, poulet, rate, histopathologie, cycle cellulaire, apoptose

## Introduction

In the poultry industry, ammonia (NH<sub>3</sub>) is considered as the greatest factor for environmental pollution. In birds, dietary protein is metabolized to uric acid, which can be ultimately converted to NH<sub>3</sub> under suitable conditions, depending on the moisture content and pH of the diet (Hong et al. 2012), ambient temperature (Calvet et al. 2011), ventilation

rate (Zhao et al. 2015), and bird age (Almuhanna et al. 2011). Previous studies have shown that NH<sub>3</sub> produced in poultry houses induced several health problems in humans, poultry, and the environment (Naseem and Annie 2018). NH<sub>3</sub> induced acute and chronic effects on poultry workers' health because of the direct irritant of NH<sub>3</sub> and the production of PM<sub>2.5</sub> (Kirychuk et al. 2003). Generally, the recommended concen-

**Table 1.** Composition and nutrient levels of the basal diet (air-dry basis) (g/kg).

Ingredients	Content	Nutrient levels	Content
Corn	553	ME (MJ/kg)	12.13
Soybean meal	365.7	CP	205
Admixture oil	28	Lys	11.5
Limestone	13.1	Met + Cys	8.1
CaHPO <sub>4</sub>	12.6	Ca	9
NaCl	12.6	TP	5.7
Premix <sup>a</sup>	3		
50% choline (50%)	10		
Lys	0.2		
Met	2.4		

<sup>a</sup>The premix provided the following per kg of the diet: Cu, 8 mg; Fe, 90 mg; Zn, 50 mg; Mn, 80 mg; I, 0.30 mg; Se, 0.15 mg; vitamin A (retinol), 10 000 IU; vitamin D<sub>3</sub> (cholecalciferol), 2100 IU; vitamin E (tocopherol), 14.97 IU; vitamin K<sub>3</sub> (menaphthone), 0.6 mg; vitamin B<sub>1</sub> (thiamine), 2.0 mg; vitamin B<sub>2</sub> (riboflavin), 4.0 mg; vitamin B<sub>12</sub> (cyanocobalamin), 0.01 mg; nicotinic acid, 30.0 mg; folic acid, 0.6 mg; biotin, 0.15 mg; D-pantothenic acid, 11 mg; phytase, 700 U.

tration of NH<sub>3</sub> in poultry houses is less than 25 ppm (Green et al. 2008). Atmospheric NH<sub>3</sub> at levels of 20–200 ppm could cause a reduction in growth performance (Shu et al. 2020), injuries to the chicken's nasal cavity and eyes, and suppression of immune function, and higher NH<sub>3</sub> levels even resulted in painful burns on legs and feet (Aziz and Barnes 2010).

It was found that atmospheric NH<sub>3</sub> caused immunosuppression in poultry, shown by the increased weight of the bursae of Fabricius (Wei et al. 2015), reduced specific antibody titers (Wang et al. 2010), increased disease susceptibility (Beker et al. 2004), increased IL6 and IL10 in plasma, and up-regulated gene expression of various cytokines in the spleen (Wu et al. 2017). Spleen is the largest peripheral immune organ, which is related to the humoral and cellular immune function of the body. Therefore, we put forward a hypothesis that ammonia stress can cause splenic damage. In the present research, we studied the effects of ammonia on the histological structure of the spleen, and tried to clarify its pathogenic mechanism related to apoptosis and proliferation.

## Materials and methods

### Animals, diets, and experimental design

The experiment was performed in the farm of the Animal Nutrition Institute of Sichuan Agricultural University, China. All of the experimental procedures were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University (Ethical code: DYY-2018203007).

In this experiment, 240 female broilers (1 day old, Cobb 500 strain), obtained from the Poultry Breeding Farm of Sichuan Agricultural University, were randomly divided into two treatments (control group and NH<sub>3</sub> group). There were six replicates per treatment and 20 chickens per replicate. The basal diet (Table 1) was formulated based on the requirements recommended by the Feeding Standard of Chicken (Ministry of Agriculture of the People's Republic of China 2004). The chickens in each replicate were raised in envi-

ronmentally controlled chambers (4500 mm × 3000 mm × 2500 mm). In the NH<sub>3</sub> group, the ammonia flowed from the air inlet at the bottom of the chamber, and the concentration of NH<sub>3</sub> in the chamber was 70 ± 5 ppm. In the control group, the concentration of NH<sub>3</sub> was below 5 ppm. The level of NH<sub>3</sub> was selected based on the NH<sub>3</sub> concentration in poultry coop during winter, as it could have caused severe damage in chickens (Naseem and Annie 2018; Shu et al. 2020). The ventilation, photoperiod, temperature, and relative humidity were automatically maintained at the same level in the two groups according to the broilers' management guidelines (Zheng et al. 2019). The NH<sub>3</sub> concentration in the chambers was monitored using LumaSense Photoacoustic Field Gas Monitor Innova 1412 (Santa Clara, CA, USA). The temperature was maintained at 32 °C during the first 2 days. And then, the ambient temperature was gradually decreased by 1 °C every 2 days. All birds were housed under a 16 h light and 8 h dark cycle and the feed and water were provided ad libitum. The relative humidity of the house was maintained at 50%. After acclimation for 1 day (day 1), the broilers were treated with different atmospheric conditions from days 2 to 8.

On days 2 and 8, the chickens and remaining feed were weighed for each replicate to determine BW gain (BWG), final body weight (FBW), and feed intake (FI) during days 2–8. Feed conversion ratio (FCR) was calculated as follows: FCR = BWG/FI. The clinical symptoms were observed during this period. By day 8, 12 birds with body weight being close to the average in each group were randomly selected and humanely executed by injecting phenobarbital sodium intravenously at a dose of 100 mg/kg, and the remaining chickens were raised by the farm owner after the experiment. Then, spleens were sampled for determining the belowmentioned parameters.

### Relative weight of spleen

After measuring the body weight, 12 birds in each group were euthanized and necropsied. The spleen was weighed after dissecting connective tissue around the organ. The relative weight of the spleen was calculated by the following formula:

$$\text{Relative weight} = \text{organ weight (g)}/\text{body weight (kg)}$$

### Pathological observation

The macroscopic changes of the spleens were observed and photographed. Then, they were fixed in 4% paraformaldehyde and routinely processed in paraffin. The 5 μm thick sections were stained with hematoxylin and eosin Y, and typical histopathological changes were pictured with a digital camera (Leica, Wetzlar, Germany).

The spleens were fixed in 2.5% glutaraldehyde and embedded in araldite. The 65–75 nm thick sections stained with uranyl acetate and poststained with 0.2% lead citrate were used for observing ultrastructural lesions by an electron microscope (Hitachi, Tokyo, Japan).

### Cell cycle of spleen by flow cytometry

The spleens were dissected in pieces and filtered through a 300-mesh nylon gauze. Then, a single-cell suspension at a

concentration of  $1 \times 10^6$  cells/mL was prepared in 4 °C phosphate buffer solution. Five hundred microliters of cell suspension was permeabilized with 1 mL of 0.25% Triton X-100 at 4 °C for 20 min. After these cells were stained with 5  $\mu$ L PI/RNase Staining Buffer (BD Pharmingen, CA, USA) for 30 min at 4 °C, the proportion of splenocytes in G<sub>0</sub>G<sub>1</sub>, S, and G<sub>2</sub>M phases was measured by flow cytometry (FCM, CytoFLEX, Beckman Coulter Inc., Germany) within 45 min and analyzed by ModFit software (Verity Software House, Maine, USA).

### Annexin V apoptosis detection by FCM

Briefly, 100  $\mu$ L single-cell suspension was mixed with 5  $\mu$ L Annexin V–fluorescein isothiocyanate and 5  $\mu$ L propidium iodide (Invitrogen, CA, USA). After the mixture was incubated at 25 °C for 15 min in the dark, 400  $\mu$ L of  $1 \times$  binding buffer was added. The apoptosis percentage was determined within 1 h by FCM, and analyzed by Kaluza 2.1 software (Beckman Coulter Inc., Germany).

### TUNEL assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed using an In Situ Cell Apoptosis Detection Kit (Boster, Wuhan, China), according to the instructions of the manufacturer and a previous description (2011). Five fields of each slice were photographed for analyzing the average optical density (AOD) in each field.

### Intracellular reactive oxygen species (ROS) detection by FCM

Intracellular ROS was measured using a commercial kit (Thermo Fisher Scientific, MA, USA). Three hundred microliters of single-cell suspension was added into 5  $\mu$ L 2,7-dichlorodihydrofluorescein diacetate. And then, the mixture was incubated at 37 °C for 20 min in the dark. Final analysis by FCM was conducted within 1 h.

### Mitochondrial membrane potential (JC-1) detection by FCM

A MitoScreen kit (JC-1; BD Pharmingen, CA, USA) was used to determine mitochondrial membrane potential (MMP). One microliter of single-cell suspension was mixed with 0.5 mL JC-1 working solution. The mixture was incubated for 15 min at 37 °C in a 5% CO<sub>2</sub> incubator. After the cells were washed twice and resuspended, MMP was assayed by FCM within 30 min.

### Quantitative real-time PCR

The total RNA was extracted from spleen tissues according to the instructions of the RNAiso Plus kit (TaKaRa, Dalian, China). The concentration of extracted total RNA was measured by spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific Inc.), and then cDNA was reverse-transcribed with a Transcriptor First Strand cDNA Synthesis kit (Roche, Basel, Switzerland). The real-time PCR reactions were performed in 20  $\mu$ L reactions, and the conditions of reactions were 2 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C, and a final melting curve analysis. All samples were conducted in triplicate on a CFX96 Touch™ Real-Time PCR

Detection System (Bio-Rad, Hercules, CA, USA). The primer sequences are displayed in Table S1. The  $\beta$ -actin was selected as the housekeeping gene because of its stability of expression. Relative abundance of mRNA of target gene was calculated using the  $2^{-\Delta\Delta CT}$  method.

### Bax, Bcl-2, caspase 3, and PCNA detected by IHC method

An streptavidin-peroxidase (SP) kit (ZSGB-BIO, Beijing, China) was used for detecting the expression of Bax, Bcl-2, caspase 3, and PCNA, according to the instructions of the manufacturer. Immunohistochemistry (IHC) was performed using the following antibodies: mouse anti-Bax (GeneTex, Southern California, USA), rabbit anti-Bcl-2 (Proteintech, Wuhan, China), rabbit anti-caspase 3, and mouse anti-proliferating cell nuclear antigen (anti-PCNA) (ABCam, Cambridge, UK). The AOD values were analyzed using Image Pro Plus 6.0 software (Media Cybernetics, Inc.; Bethesda, MD, USA).

### Detection of MDA content and T-AOC in the spleen

The spleen tissues were homogenized by using a cell homogenizer (Bio-Gen Pro200, PRO Scientific Inc., Atkinson, NH, USA). Total protein concentration of the supernatant was detected using a BCA Protein Assay Kit (Beyotime, Shanghai, China). Using the commercial kits (Nanjing Jiancheng Bio-engineering Institute Co., Nanjing, China), malondialdehyde (MDA) content and total antioxidant capacity (T-AOC) content were determined. The results were expressed as nanomoles (nM) for MDA and millimoles (mM) for T-AOC.

### Statistical analysis

Statistical analysis was performed with SPSS 26.0, and the results were shown as means  $\pm$  standard error (M  $\pm$  SE). Statistical analyses were done using an independent samples *t* test. The difference between two groups was considered significant when  $P < 0.05$  and extremely significant when  $P < 0.01$ .

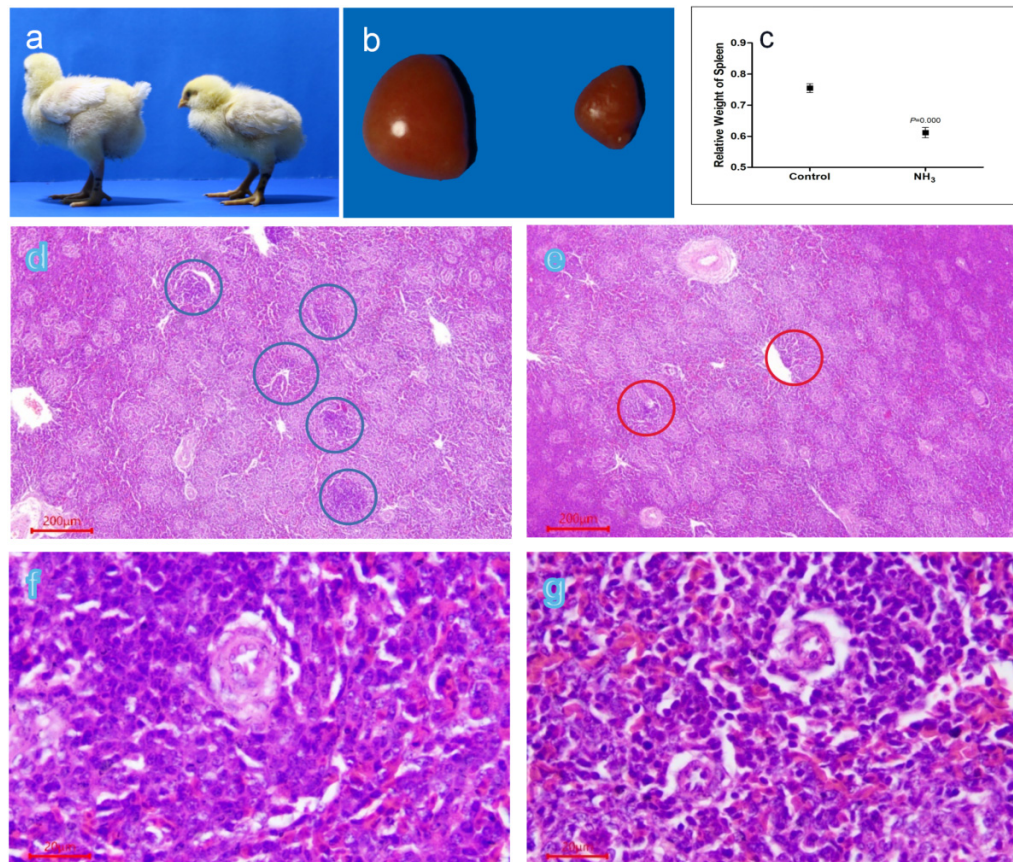
## Results

### Clinical symptoms, growth performance, and relative weight of the spleen

Chickens in the control group had normal spirit, good appetite, and perfect growth performance. However, birds in the NH<sub>3</sub> group showed decreased appetite, runny nose, and lung rales, and some of them presented excessive excitement, showing decreased pecking time and increased walking time in cages. Smaller body size was found in the NH<sub>3</sub> group (Fig. 1a). Ammonia exposure decreased the FBW, BWG, and FI ( $P < 0.05$ ) compared with those of the control group during days 2–8 (Table 2).

By gross observation, the spleen had an obvious smaller volume (Fig. 1b), and the relative weight of the spleen was significantly lower ( $P < 0.05$ ) in the NH<sub>3</sub> group (Fig. 1c).

**Fig. 1.** Comparison of clinical symptoms, relative weight, and histopathological changes of the spleen between the control group (without NH<sub>3</sub> challenge) and the NH<sub>3</sub> group (70 ± 5 ppm NH<sub>3</sub>) at day 8 of the experiment. (a) The chicken from the NH<sub>3</sub> group (the right one) has a smaller body size. (b) The spleen in the NH<sub>3</sub> group (the right one) is smaller. (c) The relative weight of the spleen in the NH<sub>3</sub> group is significantly lower than that in the control group ( $P < 0.01$ ). The short lines on the vertical bar represent the standard error. Panels (d–g) present the histological characteristics of the spleen. Panel (d) shows that there are several obvious white pulps in the spleen of the control group. (e) There are only a few small clumps of lymphocytes in the spleen of NH<sub>3</sub> group. Under high magnification, there is a smaller white pulp with loosely arranged lymphocytes in the NH<sub>3</sub> group (g) than that in the control group (f). [Colour online.]



**Table 2.** Effects of ammonia exposure on the growth performance of broilers.

Group	IBW <sup>a</sup> (g)	FBW (g)	BWG <sup>a</sup> (g)	FI <sup>a</sup> (g)	FCR <sup>a</sup> (g:g)
Control <sup>b</sup>	42.97 ± 0.15	157.25 ± 1.28	16.69 ± 0.29	139.69 ± 4.71	1.22 ± 0.03
NH <sub>3</sub> <sup>b</sup>	42.99 ± 0.12	140.63 ± 6.75	13.95 ± 0.96	114 ± 4.45	1.18 ± 0.04
P	0.096	0.005	0.021	0.032	0.091

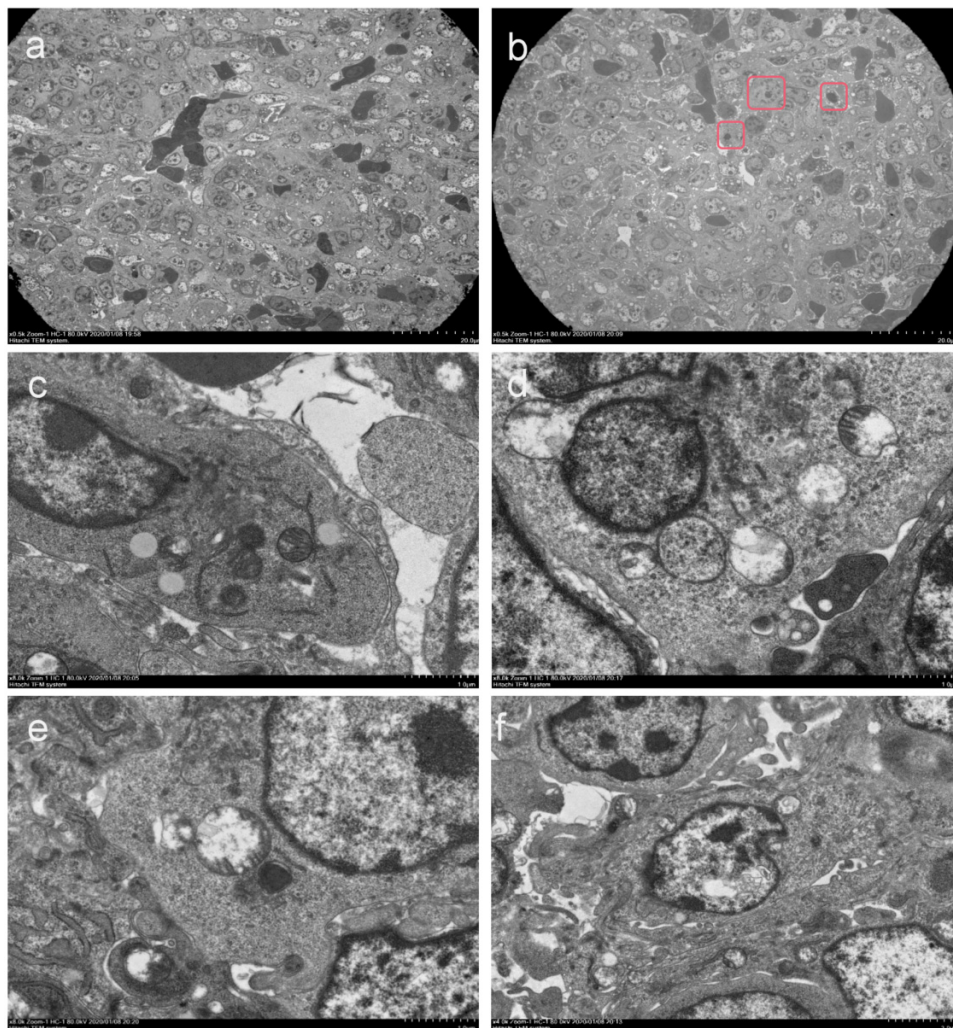
<sup>a</sup>IBW and FBW, initial body weight and final body weight, respectively; BWG, body weight gain; FI, feed intake; FCR, the ratio of feed consumption and body weight gain. These indicators were measured on days 2 and 8 of the experiment. The numbers after “±” indicate the standard deviation. <sup>b</sup>Control and NH<sub>3</sub> represented the control group with the normal atmosphere and the NH<sub>3</sub> group treated with 70 ± 5 ppm NH<sub>3</sub> ammonia, respectively.

## Histopathological and ultrastructural changes of the spleen

Histopathological observation indicated the lesions of the spleen in the NH<sub>3</sub> group. The white pulps were dysplastic. At low magnification, several obvious white pulps could be found in the control group. However, no typical white pulp and only a few small clumps of lymphocytes could be observed in the NH<sub>3</sub> group. At high magnification, the lymphocytes in the white pulp were sparsely arranged (Fig. 1f and 1g).

Ultrastructural pathological observation showed that the subcellular structure was normal in the splenocytes of the control group (Fig. 2a and 2c). In the NH<sub>3</sub> group, the apoptotic cells with concentrated chromatin in the center or margin could be more easily found (Fig. 2b). The mitochondria of reticuloendothelial cells and lymphocytes were swollen obviously, whose ridges were broken and dissolved into vacuoles (Fig. 2d). The endoplasmic reticulum (ER) of lymphocytes was expanded and accumulated substances with high

**Fig. 2.** Comparison of ultrastructural characteristics of the spleen between the control group (without NH<sub>3</sub> challenge) and the NH<sub>3</sub> group (70 ± 5 ppm NH<sub>3</sub>) on day 8 of the experiment. Compared with the control group (a), the apoptotic cells with concentrated chromatin in the center or margin of some splenocytes (red box) can be found in the NH<sub>3</sub> group (b). Under high magnification, the splenocytes of the control group are normal (c). In the NH<sub>3</sub> group, swollen mitochondria of a splenocyte (d), expanded ER of lymphocyte (e), and membrane inclusions in the nucleus of lymphocyte (f) can be seen. [Colour online.]



electron density (Fig. 2e). Sometimes, membrane inclusions were observed in the nucleus of lymphocytes (Fig. 2f).

### Cell cycle phase distribution of splenic cells and expression of cyclins

Compared with those of the control group, the G<sub>0</sub>G<sub>1</sub> phase cell distribution was significantly increased ( $P < 0.05$ ), but the G<sub>2</sub>M phase cell distribution and proliferation index (PI) of the splenocytes were decreased in the NH<sub>3</sub> group ( $P < 0.05$ ). There were no significant differences in the S phase cell distribution between the two groups ( $P > 0.05$ ). Figure 3a–3c visually displays the decrease of the G<sub>2</sub>M peak.

The nucleus of PCNA-positive cells was brown-stained in the NH<sub>3</sub> group (Fig. 3e) and the control group (Fig. 3d). The AOD value of PCNA-positive materials in the NH<sub>3</sub> group was significantly lower ( $P < 0.05$ ) than that in the control group (Fig. 3f).

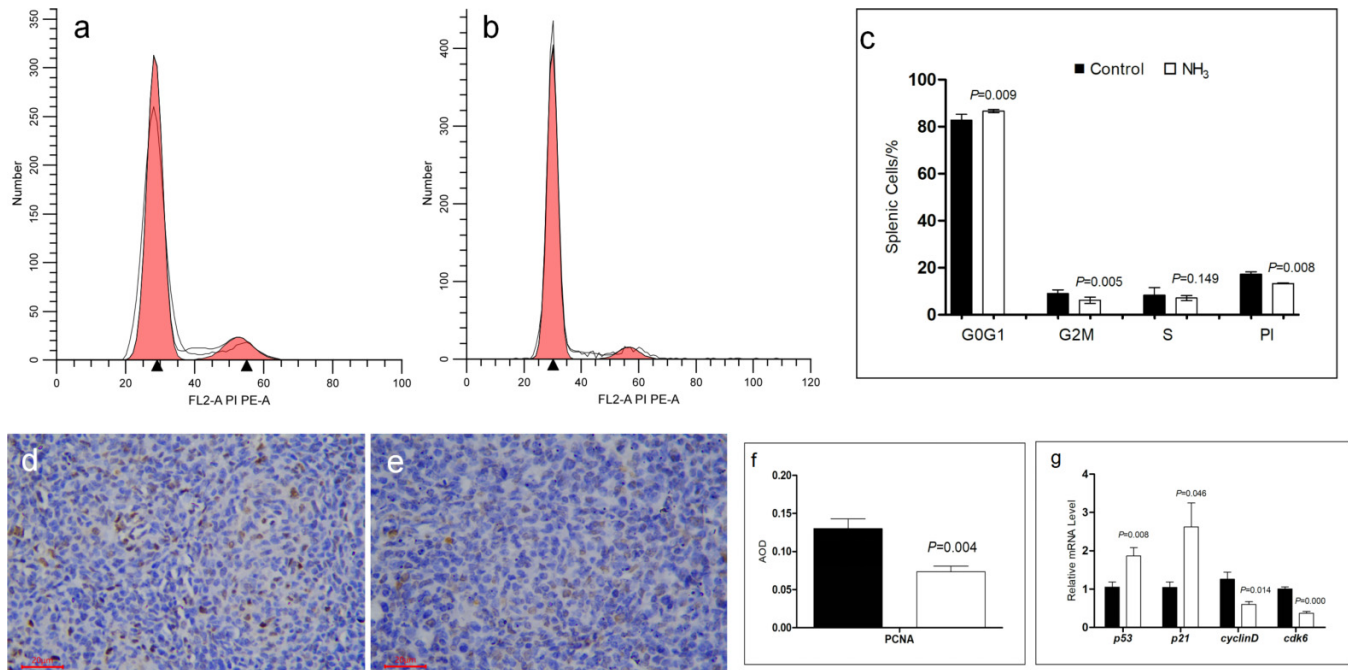
According to the qPCR analysis of relative expressions of cyclin genes, the upregulated expressions of P53 and P21, and downregulated expressions of cyclin D1 and CDK6 were observed in the NH<sub>3</sub> group ( $P < 0.05$ ) compared with the control group (Fig. 3g).

### Apoptotic percentage and expression of apoptosis regulator

By FCM, the percentage of apoptotic splenocytes in the NH<sub>3</sub> group was significantly higher ( $P < 0.01$ ) than that in the control group (Fig. 4a, 4b, and 4g). The results of the TUNEL staining were similar, and the AOD values of the positive apoptotic cells were significantly increased ( $P < 0.05$ ) in the NH<sub>3</sub> group (Fig. 4f, 4k, and 4r).

Apoptosis is frequently associated with depolarization of the MMP ( $\Delta\psi_m$ ), which can be presented as a lower red fluorescence signal intensity of JC-1. The results indicated that the percentage of cells with lower red fluorescence in the NH<sub>3</sub>

**Fig. 3.** Comparison of the cell cycle phase distribution of splenic cells and the expression of cyclins in the spleen between the control group (without NH<sub>3</sub> challenge) and the NH<sub>3</sub> group (70 ± 5 ppm NH<sub>3</sub>) at day 8 of the experiment. Note: Showing the parameters related to the cell cycle. The percentage of G<sub>0</sub>G<sub>1</sub> phase cells is higher ( $P < 0.05$ ) and G<sub>2</sub>M phase cell distribution and PI are lower ( $P < 0.05$ ) in the NH<sub>3</sub> group than in the control group (c). On the histogram from FCM detection, the decrease of G<sub>2</sub>M peak height is shown directly in the NH<sub>3</sub> group (b) compared with the control group (a). By the IHC method, there are fewer PCNA positive cells in the NH<sub>3</sub> group (e) than in the control group (d), and the OD values of positive materials were lower in the NH<sub>3</sub> groups too (f). The short lines on the vertical bar represent the standard error. [Colour online.]



group was significantly higher than that in the control group ( $P < 0.05$ ) (Fig. 4c, 4d, and 4h). ROS kit was used to detect the percentage of splenic cells with ROS. The result showed that the percentages of positive cells in the NH<sub>3</sub> group were higher ( $P < 0.05$ ) (Fig. 4e, 4f, and 4i).

The IHC method was performed to judge the expression of tissue Bax, Bcl-2, and caspase 3. The cytoplasm of positive cells was brownish yellow (Fig. 4l–4q). By determining the AOD values, the results revealed an increased expression of Bax and caspase 3 ( $P < 0.01$ ) and a decreased expression of Bcl-2 ( $P < 0.01$ ) in the NH<sub>3</sub> group compared to the control group. At the same time, the ratio of Bax/Bcl-2 was increased significantly ( $P < 0.05$ ) (Fig. 4s).

According to the qPCR analysis, the expressions of Bax and caspase 3 and the ratio of Bax/Bcl-2 were significantly increased in the NH<sub>3</sub> group ( $P < 0.05$ ). However, the expressions of Bcl-2 were significantly downregulated in the NH<sub>3</sub> group ( $P < 0.05$ ) (Fig. 4s).

### Detection of MDA content and T-AOC

As shown in Fig. 4s, the contents of MDA were significantly increased ( $P < 0.05$ ), and the T-AOC was decreased ( $P = 0.057$ ) in the NH<sub>3</sub> group compared with the control group.

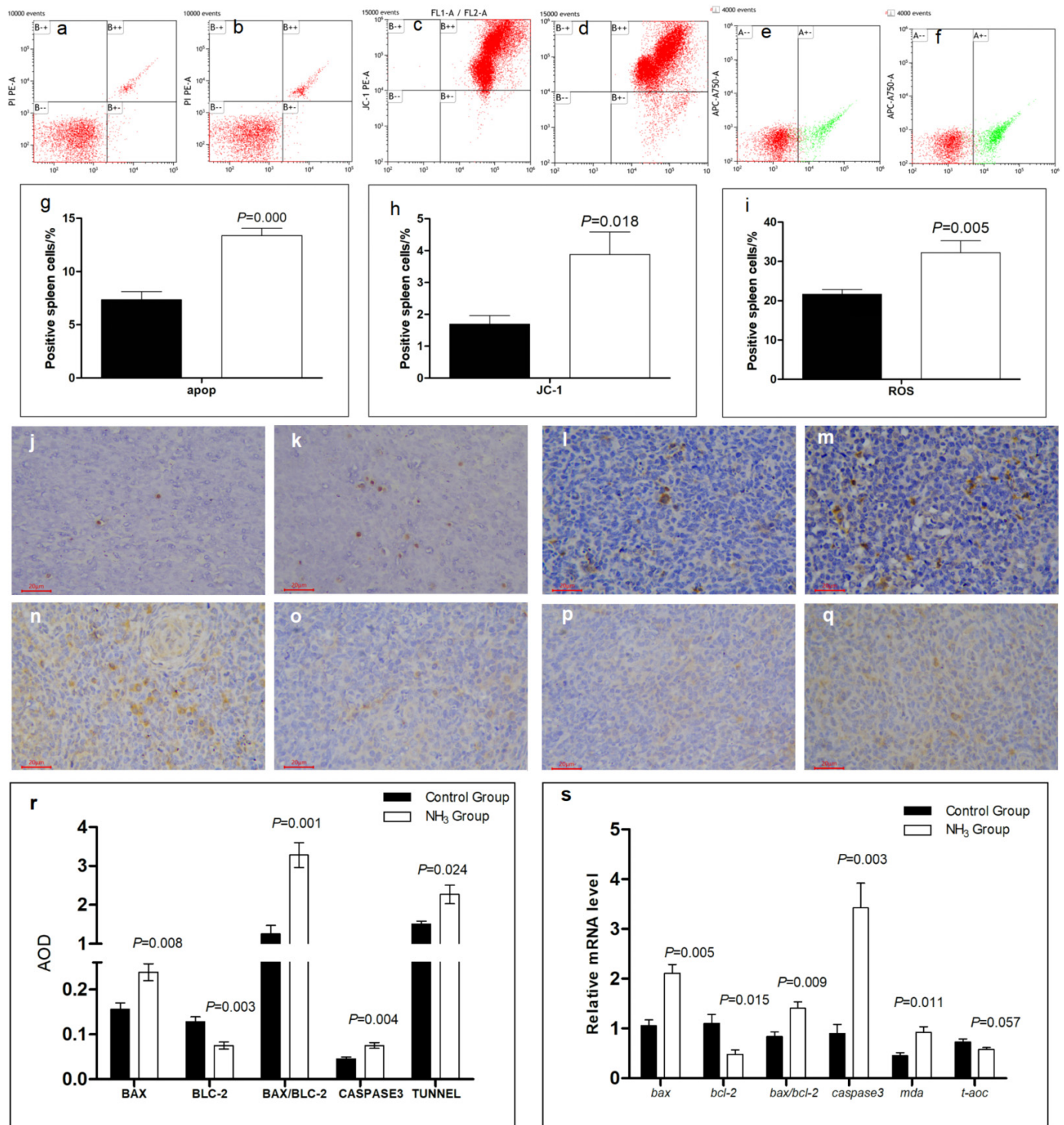
## Discussion

According to a review paper, atmospheric NH<sub>3</sub> at levels of 20–200 ppm could result in respiratory symptoms and poor growth performance in poultry (Aziz and Barnes 2010). Similarly, we observed that 70 ± 5 ppm NH<sub>3</sub> caused obvious respiratory signs, including runny nose and lung rales, retarded growth showing as lower DWG, and higher FCR in broilers. Excessive excitement, a clinical sign that has never been reported before, hinted at the neurotoxicity of NH<sub>3</sub> that needs to be further explored.

In mammals and birds, the spleen is the principal peripheral immune organ, involving cellular immunity and humoral immunity. So far, the spleen histological lesions caused by atmospheric NH<sub>3</sub> have not been reported, and the gross lesions of immune organs caused by ammonia exposure were limited to the description of bursa of Fabricius (Shah et al. 2020). In the present study, we observed the anatomical, histopathological, and ultrastructural changes of the spleen in broilers exposed to 70 ± 5 ppm NH<sub>3</sub>. The results showed that the development of the spleen could be inhibited by NH<sub>3</sub> exposure, shown by the decrease of relative weight, dysplasia of white pulps, and expansion of mitochondria and ER in lymphocytes.

Cell cycle, including G<sub>0</sub>, G<sub>1</sub>, S, G<sub>2</sub>, and M phases, is the process by which one cell divides into two. Two main cell cycle

**Fig. 4.** Comparison of the apoptotic percentage and the expression of apoptosis regulator in the spleen between the control group (without NH<sub>3</sub> challenge) and the NH<sub>3</sub> group (70 ± 5 ppm NH<sub>3</sub>) at day 8 of the experiment. A1–A6 are the quadrant diagrams of apoptosis-related parameters detected by FCM, which in pairs present the percentages of apoptotic splenocytes by Annexin V staining (*a, b*), the number of cells with lower red fluorescence by JC-1 staining (*c, d*), and the percentages of ROS-positive cells (*e, f*). By statistically analyzing, panels (*g, h, and i*) show the significant increase of apoptotic cells, low red fluorescence cells, and ROS-positive cells, respectively ( $P < 0.05$ ). From panels (*j–q*), the histological pictures in pairs show the expression of TUNEL, Bax, Bcl-2, and caspase 3, respectively, by IHC methods. Panel (*r*) shows the significant increase of apoptotic cells, positive expressions of Bax and caspase 3, and the obvious decrease of Bcl-2. The short lines on the vertical bar represent the standard error. [Colour online.]





checkpoints at the G<sub>1</sub>/S phase and the G<sub>2</sub>/M phase are often used to monitor and regulate the progress of the cell cycle (Li et al. 2020). In the present experiment, the results showed that the percentage of splenocytes during the resting period (G<sub>0</sub>/G<sub>1</sub> phase) was increased and the percentages of cells during the dividing period (G<sub>2</sub>/M) were decreased, which could result in the retarded growth of the spleen presenting as decreased relative weight. Its molecular mechanism was further explored by evaluating the protein and mRNA expressions of some cycle regulators.

PCNA was demonstrated to be present in proliferating cells (Strzalka and Ziemienowicz 2011). In the present study, the AOD value of PCNA-positive cells in the NH<sub>3</sub> group was lower, indicating that NH<sub>3</sub> could inhibit the proliferation of the spleen. The process of cell cycle is mainly regulated by cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CKIs) (Leal-Esteban and Fajas 2020). Cyclin D1 forms a complex with CDK to promote cell proliferation by urging the transformation from G<sub>1</sub> phase to S phase (Huber et al. 2021). However, CDK activity is regulated by CKI (Bury et al. 2021). P21, as a CKI, prevents cells from entering the S phase by inhibiting the activity of PCNA and the synthesis of DNA (Hobeika et al. 1999). While the DNA suffers from irreversible damage, P53 protein will regulate the cycle signaling pathway, leading to cell cycle stagnation, apoptosis and senescence (Stewart and Weinberg 2006). Our results showed that the relative expressions of cyclin D1 and CDK6 mRNA were significantly downregulated, while the relative expressions of P21 and P53 mRNA were significantly upregulated under ammonia stress. Therefore, the two factors, namely, the decreased expression of CDK genes and the increased expression of CKI genes, coordinately led to the failure of the transition from G<sub>1</sub> phase to S phase, which could further induce cell cycle blockage at G<sub>0</sub>/G<sub>1</sub> phase, inhibit proliferation, or promote apoptosis of splenic cells.

Apoptosis is a programmed death procedure that is involved in the development of immune organs (Hu et al. 2018). It has been found that various harmful factors, such as drugs, waste gas, and pathogens, could cause upregulation of splenocytes in human beings, mice, and birds (Weinrauch and Zychlinsky 1999; Elmore 2007). Considering the effect of NH<sub>3</sub> on immunity, it was reported that NH<sub>3</sub> exposure induced the increase of apoptosis in the bursa of Fabricius and liver (Shah et al. 2020; Xu et al. 2020). In the present study, atmospheric NH<sub>3</sub> at 70 ± 5 ppm caused an increase of apoptotic splenocytes, which may be one of the reasons for the delayed growth of the spleen. Its molecular mechanism was further explored by evaluating the protein and mRNA expressions of some apoptotic regulatory proteins and indicators reflecting the equilibrium state of redox.

It is well accepted that oxidative stress is an apoptosis inducer (Marin and Taranu 2012). Because of redox imbalance, a large number of ROS are produced in cells, and cause the loss of MMP, activating mitochondrial pathways to induce apoptosis (Cheng et al. 2016). The decrease of MMP was considered to be an early case in the activation of the mitochondria apoptotic pathway (Cheng et al. 2016). In the present study, the increased percentage of splenocytes with ROS, the decreased activity of T-AOC, and the increased concentra-

tion of MDA suggested that the oxidative stress in the spleen could be triggered by NH<sub>3</sub> exposure in broilers. To judge the condition of MMP depolarization, JC-1 staining was used. The results showed that the percentage of splenocytes with MMP depolarization was significantly higher in chicks exposed to 70 ± 5 ppm NH<sub>3</sub>. When MMP depolarization occurs, membrane permeability is enhanced, which may cause mitochondrial swelling and release of proapoptotic factors, finally leading to apoptosis (Liu and Wang 2016). In our study, mitochondrial swelling was also found by ultrastructural observation. By qPCR determination, we found that the protein and mRNA expressions of Bax and caspase-3 were increased, and the protein and the mRNA expression of Bcl-2 was decreased in the NH<sub>3</sub> group, which were correlated with the activation of the mitochondrial pathway. Our results suggested that splenic apoptosis might be triggered through the mitochondrial pathway related to oxidative stress.

## Conclusion

Taken together, our results showed that ammonia exposure was able to induce a series of events in the spleen of chickens, including a decrease of relative weight, dysplasia of white pulps, G<sub>0</sub>/G<sub>1</sub> phase arrest of the cell cycle, and excessive apoptosis by touching off mitochondria-mediated apoptotic pathways. The results suggested that NH<sub>3</sub> exposure could induce spleen lesions in chickens, and its possible mechanisms were cell cycle arrest and apoptosis activation.

## Acknowledgements

This work was supported by the International Fund of State Forestry Administration (2017) and the National Natural Science Foundation of China under Grant No. 2016YFD0500504.

## Article information

### History dates

Received: 25 August 2021

Accepted: 19 February 2022

Accepted manuscript online: 23 March 2022

Version of record online: 11 August 2022

### Copyright

© 2022 Sichuan Agricultural University, Sichuan Youngster Technology Co., Shaoguan University, and Chengdu University. This work is licensed under a [Creative Commons Attribution 4.0 International License](https://creativecommons.org/licenses/by/4.0/) (CC BY 4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.

## Author information

### Author notes

Tong Cai, Cuiyan Zhao, and Lin Chen contributed equally to this paper.

## Competing interests

The authors have declared that no competing interests exist.

## Supplementary material

Supplementary data are available with the article at <https://doi.org/10.1139/CJAS-2021-0084>.

## References

- Almuhanna, E.A., Ahmed, A.S., and Yousif, Y.M.A. 2011. Effect of air contaminants on poultry immunological and production performance. *Int. J. Poult. Sci.* **10**: 461–470. doi:10.3923/ijps.2011.461.470.
- Aziz, T., and Barnes, H.J. 2010. Harmful effects of ammonia on birds. *Poult. World*, **26**: 28–30.
- Beker, A., Vanhooser, S.L., Swartzlander, J.H., and Teeter, R.G. 2004. Atmospheric ammonia concentration effects on broiler growth and performance. *J. Appl. Poult. Res.* **13**: 5–9. doi:10.1093/japr/13.1.5.
- Bury, M., Le Calvé, B., Ferbeyre, G., Blank, V., and Lessard, F. 2021. New insights into CDK regulators: novel opportunities for cancer therapy. *Trends Cell Biol.* **31**(5): 331–344. doi:10.1016/j.tcb.2021.01.010. PMID: 33676803.
- Calvet, S., Cambra-López, M., Estelles, F., and Torres, A.G. 2011. Characterization of gas emissions from a Mediterranean broiler farm. *Poult. Sci.* **90**: 534–542. doi:10.3382/ps.2010-01037. PMID: 21325223.
- Cheng, F., Lan, J., Xia, W., Tu, C., Chen, B., Li, S., and Pan, W. 2016. Folic acid attenuates vascular endothelial cell injury caused by hypoxia via the inhibition of ERK1/2/NOX4/ROS pathway. *Cell Biochem. Biophys.* **74**: 205–211. doi:10.1007/s12013-016-0723-z. PMID: 26906511.
- Elmore, S. 2007. Apoptosis: a review of programmed cell death. *Toxicol. Pathol.* **35**: 495–516. doi:10.1080/01926230701320337.
- Green, A.R., Wathes, C.M., Demmers, T.G., Macarthur, C.J., and Xin, H. 2008. Development and application of a novel environmental preference chamber for assessing responses of laboratory mice to atmospheric ammonia. *J. Am. Assoc. Lab. Anim. Sci.* **47**: 49–56. doi:10.1111/j.1748-5827.2007.00532.x.
- Hobeika, A.C., Etienne, W., Torres, B.A., and Johnson, H.M. 1999. IFN-gamma induction of P21(WAF1) is required for cell cycle inhibition and suppression of apoptosis. *J. Interferon Cytokine Res.* **19**: 1351–1361. doi:10.1089/107999099312812. PMID: 10638704.
- Hong, L., Xin, H., Bums, R.T., Roberts, S.A., Li, S., Kliebenstein, J., and Bregendahl, K. 2012. Reducing ammonia emissions from laying-hen houses through dietary manipulation. *J. Air Waste Manage. Assoc.* **62**: 160–169. doi:10.1080/10473289.2011.638414.
- Hu, X., Chi, Q., Wang, D., Chi, X., Teng, X., and Li, S. 2018. Hydrogen sulfide inhalation-induced immune damage is involved in oxidative stress, inflammation, apoptosis and the Th1/Th2 imbalance in broiler bursa of Fabricius. *Ecotoxicol. Environ. Saf.* **164**: 201–209. doi:10.1016/j.ecoenv.2018.08.029. PMID: 30118953.
- Huber, K., Mestres-Arenas, A., Fajas, L., and Leal-Esteban, L.C. 2021. The multifaceted role of cell cycle regulators in the coordination of growth and metabolism. *FEBS J.* **288**(12): 3813–3833. doi:10.1111/febs.15586. PMID: 33030287.
- Kiryuchuk, S.P., Senthilselvan, A., Dosman, J.A., Juorio, V., Feddes, J.J.R., Willson, P., et al. 2003. Respiratory symptoms and lung function in poultry confinement workers in Western Canada. *Can. Respir. J.* **10**: 375–380. doi:10.1155/2003/109679.
- Leal-Esteban, L.C., and Fajas, L. 2020. Cell cycle regulators in cancer cell metabolism. *Biochim. Biophys. Acta Mol. Basis Dis.* **1866**(5): 165715. doi:10.1016/j.bbadis.2020.165715. PMID: 32035102.
- Li, X.L., Maab, R.H., Niab, Z.J., Thakurab, K., Cespedes-Acunac, C.L., Wangd, S., et al. 2020. Dioscin inhibits human endometrial carcinoma proliferation via G0/G1 cell cycle arrest and mitochondrial-dependent signaling pathway. *Food Chem. Toxicol.* **148**: 111941. doi:10.1016/j.fct.2020.111941. PMID: 33359023.
- Liu, Y., and Wang, W. 2016. Aflatoxin B1 impairs mitochondrial functions, activates ROS generation, induces apoptosis and involves Nrf2 signal pathway in primary broiler hepatocytes. *Anim. Sci. J.* **87**(12): 1490–1500. doi:10.1111/asj.12550. PMID: 26997555.
- Marin, D.E., and Taranu, I. 2012. Overview on aflatoxins and oxidative stress. *Toxin Rev.* **31**: 32–43. doi:10.3109/15569543.2012.730092.
- Naseem, S., and Annie, J.K., 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.* **25**: 15269–15293. doi:10.1007/s11356-018-2018-y. PMID: 29705898.
- Shah, S.W.A., Chen, J., Han, Q., Xu, Y., Ishfaq, M., and Teng, X. 2020. Ammonia inhalation impaired immune function and mitochondrial integrity in the broilers bursa of Fabricius: implication of oxidative stress and apoptosis. *Ecotoxicol. Environ. Saf.* **190**: 110078. doi:10.1016/j.ecoenv.2019.110078. PMID: 31841897.
- Shu, G., Xu, D., Ran, C., Yin, L., Lin, J., Fu, H., et al. 2020. Protective effect of dietary supplementation of *Bupleurum falcatum* L. saikosaponins exposure-induced ileum injury in broilers. *Poult. Sci.* **3**(100): 100803. doi:10.1016/j.psj.2020.10.057.
- Stewart, S.A., and Weinberg, R.A. 2006. Telomeres: cancer to human aging. *Annu. Rev. Cell Dev. Biol.* **22**: 531–557. doi:10.1146/annurev.cellbio.22.010305.104518. PMID: 16824017.
- Strzalka, W., and Ziemienowicz, A. 2011. Proliferating cell nuclear antigen (PCNA): a key factor in DNA replication and cell cycle regulation. *Ann. Bot.* **107**: 1127–1140. doi:10.1093/aob/mcq243. PMID: 21169293.
- Wang, Y.M., Meng, Q.P., Guo, Y.M., Wang, Y.Z., and Shan, T.Z. 2010. Effect of atmospheric ammonia on growth performance and immunological response of broiler chickens. *J. Anim. Vet. Adv.* **9**: 2802–2806. doi:10.3923/javaa.2010.2802.2806.
- Wei, F.X., Hu, X.F., Xu, B., Zhang, M.H., Li, S.Y., Sun, Q.Y., and Lin, P. 2015. Ammonia concentration and relative humidity in poultry houses affect the immune response of broilers. *Genet. Mol. Res.* **14**(2): 3160–3169. doi:10.4238/2015.April.10.27. PMID: 25966081.
- Weinrauch, Y., and Zychlinsky, A. 1999. The induction of apoptosis by bacterial pathogens. *Annu. Rev. Microbiol.* **53**: 155–187. doi:10.1146/annurev.micro.53.1.155. PMID: 10547689.
- Wu, Y.N., Yan, F.F., Hu, J.Y., Toker, C.M., Green, A.R., and Cheng, H.W. 2017. The effect of chronic ammonia exposure on acute-phase proteins, immunoglobulin, and cytokines in laying hens. *Poult. Sci.* **96**: 1524–1530. doi:10.3382/ps/pew454. PMID: 28379573.
- Xu, Y., Li, Z., Zhang, S., Zhang, H., and Teng, X. 2020. miR-187-5p/apaf-1 axis was involved in oxidative stress-mediated apoptosis caused by ammonia via mitochondrial pathway in chicken livers. *Toxicol. Appl. Pharmacol.* **388**: 114869. doi:10.1016/j.taap.2019.114869. PMID: 31863799.
- Zhao, Y., Shepherd, T.A., Li, H., and Xin, H. 2015. Environmental assessment of three egg production systems. Part I. Monitoring system and indoor air quality. *Poult. Sci.* **94**: 518–533. doi:10.3382/ps/peu076. PMID: 25737567.
- Zheng, S., Jin, X., Chen, M., Shi, Q., Zhang, H., and Xu, Q., 2019. Hydrogen sulfide exposure induces jejunum injury via CYP450s/ROS pathway in broilers. *Chemosphere*, **214**: 25–34. doi:10.1016/j.chemosphere.2018.09.002. PMID: 30253253.