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

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

Lin Chen[a], **Tong Cai**[b], **Cuiyan Zhao**[c], **Shiping Bai**[d], **Gang Shu**[d], **Changlin Wen**[e], **Qinkun Xu**[c], **and Xi Peng**[a]

[a]College of Food and Biological Engineering, Chengdu University, No. 2025, Chengluo Road, Chengdu, Sichuan Province, 610106, China; [b]Sichuan Youngster Technology Co. Ltd., No. 18, Bayi Road, Wenjiang District, Chengdu, Sichuan Province, 611130, China; [c]Henry Fok College of Biology and Agriculture, Shaoguan University, No. 288, University Road, Zhenjiang District, Shaoguan City, Guangdong Province, 512005, China; [d]Animal Nutrition Institute, College of Veterinary Medicine, Sichuan Agricultural University, No. 211, Huiming Road, Wenjiang District, Chengdu, Sichuan Province, 611130, China; [e]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_3) 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_3 challenge) and NH_3 group (70 ± 5 ppm NH_3). The experiment lasted for 8 days. The results showed that NH_3 exposure caused decreased relative weight ($P < 0.05$), dysplasia of white pulps, obvious ultrastructural lesions, upregulation of G_0G_1 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_3 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_3) 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_3) et NH_3 (70 ± 5 ppm NH_3). L'expérience a duré huit jours. Les résultats ont montré que l'exposition à NH_3 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 G0G1, 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_3 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_3) 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_3 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_3 produced in poultry houses induced several health problems in humans, poultry, and the environment (Naseem and Annie 2018). NH_3 induced acute and chronic effects on poultry workers' health because of the direct irritant of NH_3 and the production of PM2.5 (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
$CaHPO_4$	12.6	Ca	9
NaCl	12.6	TP	5.7
Premix[a]	3		
50% choline (50%)	10		
Lys	0.2		
Met	2.4		

[a]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_3 (cholecalciferol), 2100 IU; vitamin E (tocopherol), 14.97 IU; vitamin K_3 (menaphthone), 0.6 mg; vitamin B_1 (thiamine), 2.0 mg; vitamin B_2 (riboflavin), 4.0 ng; vitamin B_{12} (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_3 in poultry houses is less than 25 ppm (Green et al. 2008). Atmospheric NH_3 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_3 levels even resulted in painful burns on legs and feet (Aziz and Barnes 2010).

It was found that atmospheric NH_3 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 upregulated 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_3 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 environmentally controlled chambers (4500 mm × 3000 mm × 2500 mm). In the NH_3 group, the ammonia flowed from the air inlet at the bottom of the chamber, and the concentration of NH_3 in the chamber was 70 ± 5 ppm. In the control group, the concentration of NH_3 was below 5 ppm. The level of NH_3 was selected based on the NH_3 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_3 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 1×10^6 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 μL PI/RNase Staining Buffer (BD Pharmingen, CA, USA) for 30 min at 4 °C, the proportion of splenocytes in G_0G_1, S, and G_2M 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 μL single-cell suspension was mixed with 5 μL Annexin V–fluorescein isothiocyanate and 5 μL propidium iodide (Invitrogen, CA, USA). After the mixture was incubated at 25 °C for 15 min in the dark, 400 μL of 1× 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 μL 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_2 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 μL 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 β-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 Bioengineering 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 ± standard error (M ± 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_3 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_3 group (Fig. 1*a*). 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. 1*b*), and the relative weight of the spleen was significantly lower ($P < 0.05$) in the NH_3 group (Fig. 1*c*).

Fig. 1. Comparison of clinical symptoms, relative weight, and histopathological changes of the spleen between the control group (without NH_3 challenge) and the NH_3 group (70 ± 5 ppm NH_3) at day 8 of the experiment. (*a*) The chicken from the NH_3 group (the right one) has a smaller body size. (*b*) The spleen in the NH_3 group (the right one) is smaller. (*c*) The relative weight of the spleen in the NH_3 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_3 group. Under high magnification, there is a smaller white pulp with loosely arranged lymphocytes in the NH_3 group (*g*) than that in the control group (*f*). [Colour online.]

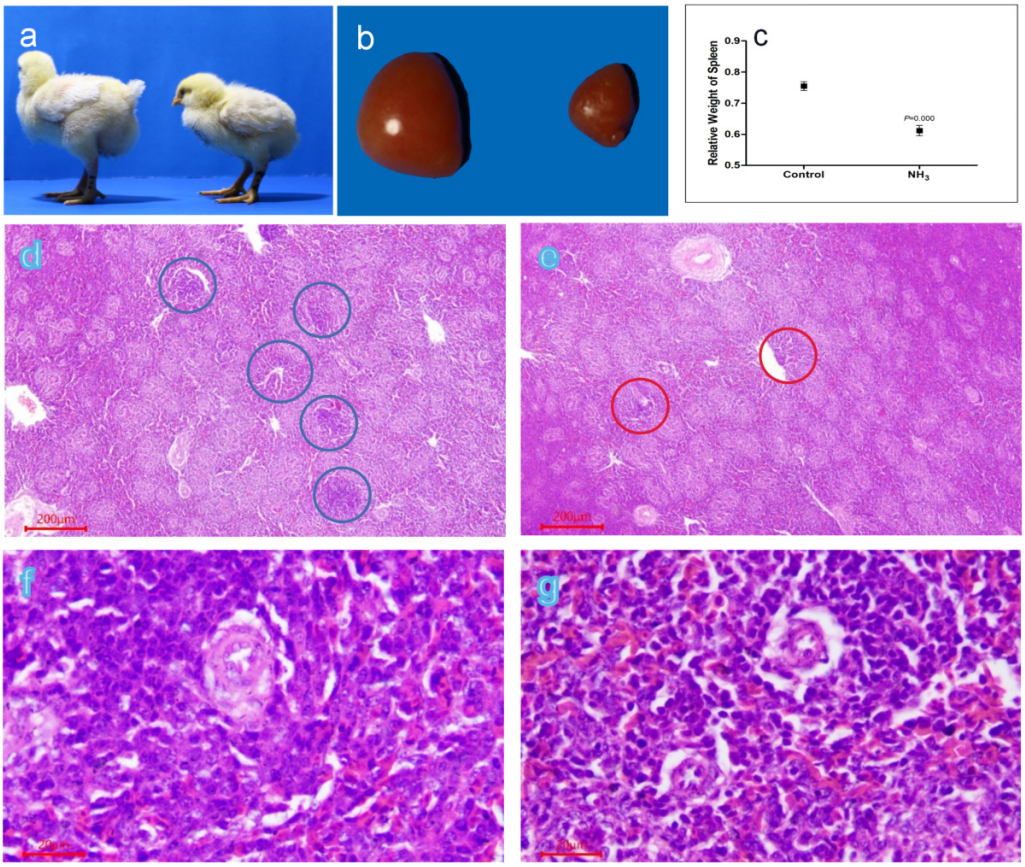


Table 2. Effects of ammonia exposure on the growth performance of broilers.

Group	IBW[a] (g)	FBW (g)	BWG[a] (g)	FI[a] (g)	FCR[a] (g:g)
Control[b]	42.97 ± 0.15	157.25 ± 1.28	16.69 ± 0.29	139.69 ± 4.71	1.22 ± 0.03
NH_3[b]	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

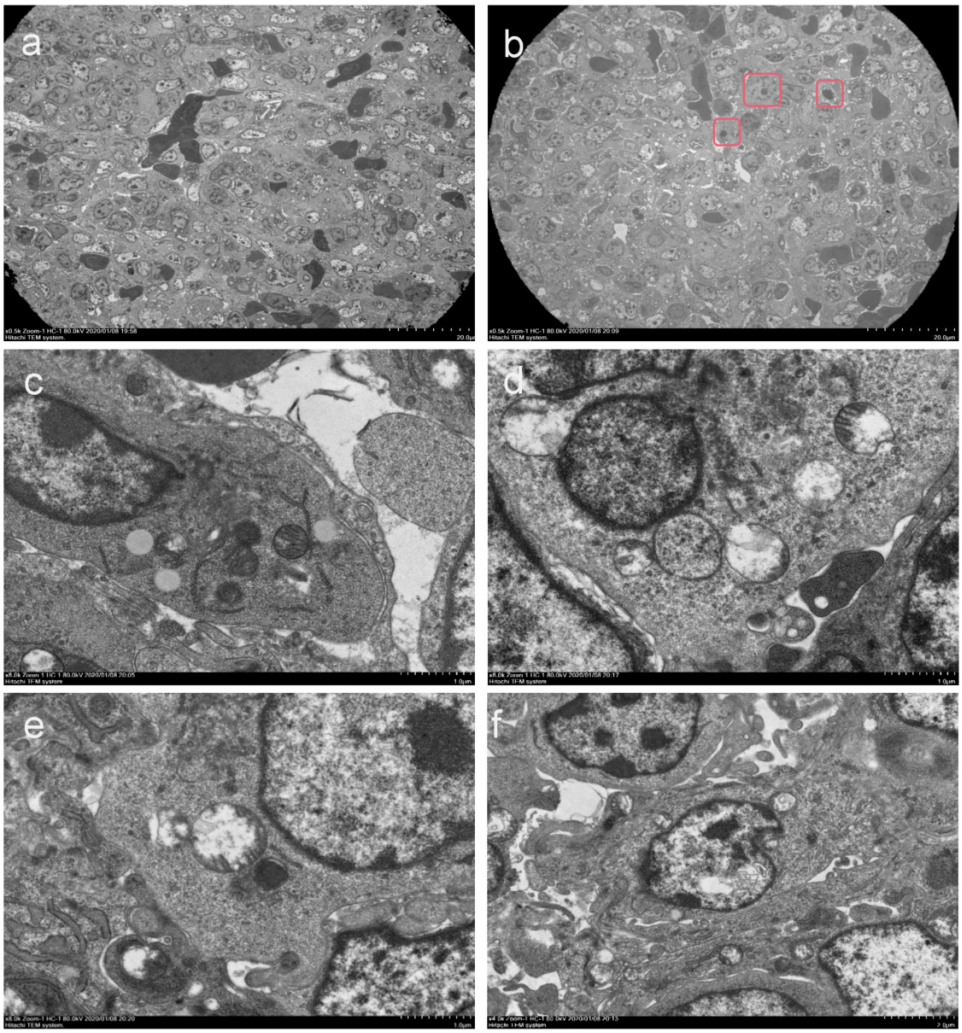
[a]IBW and FBW, initial body weight and final body weight, respectively; BWG, body weight gain; FI, feed intake; FCR, the ratio of feed consumption and body weight gain. These indicators were measured on days 2 and 8 of the experiment. The numbers after "±" indicate the standard deviation. [b]Control and NH_3 represented the control group with the normal atmosphere and the NH_3 group treated with 70 ± 5 ppm NH_3 ammonia, respectively.

Histopathological and ultrastructural changes of the spleen

Histopathological observation indicated the lesions of the spleen in the NH_3 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_3 group. At high magnification, the lymphocytes in the white pulp were sparsely arranged (Fig. 1*f* and 1*g*).

Ultrastructural pathological observation showed that the subcellular structure was normal in the splenocytes of the control group (Fig. 2*a* and 2*c*). In the NH_3 group, the apoptotic cells with concentrated chromatins in the center or margin could be more easily found (Fig. 2*b*). The mitochondria of reticuloendothelial cells and lymphocytes were swollen obviously, whose ridges were broken and dissolved into vacuoles (Fig. 2*d*). 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_3 challenge) and the NH_3 group (70 ± 5 ppm NH_3) on day 8 of the experiment. Compared with the control group (*a*), the apoptotic cells with concentrated chromatins in the center or margin of some splenocytes (red box) can be found in the NH_3 group (*b*). Under high magnification, the splenocytes of the control group are normal (*c*). In the NH_3 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. 2*e*). Sometimes, membrane inclusions were observed in the nucleus of lymphocytes (Fig. 2*f*).

Cell cycle phase distribution of splenic cells and expression of cyclins

Compared with those of the control group, the G_0G_1 phase cell distribution was significantly increased ($P < 0.05$), but the G_2M phase cell distribution and proliferation index (PI) of the splenocytes were decreased in the NH_3 group ($P < 0.05$). There were no significant differences in the S phase cell distribution between the two groups ($P > 0.05$). Figure 3*a*–3*c* visually displays the decrease of the G_2M peak.

The nucleus of PCNA-positive cells was brown-stained in the NH_3 group (Fig. 3*e*) and the control group (Fig. 3*d*). The AOD value of PCNA-positive materials in the NH_3 group was significantly lower ($P < 0.05$) than that in the control group (Fig. 3*f*).

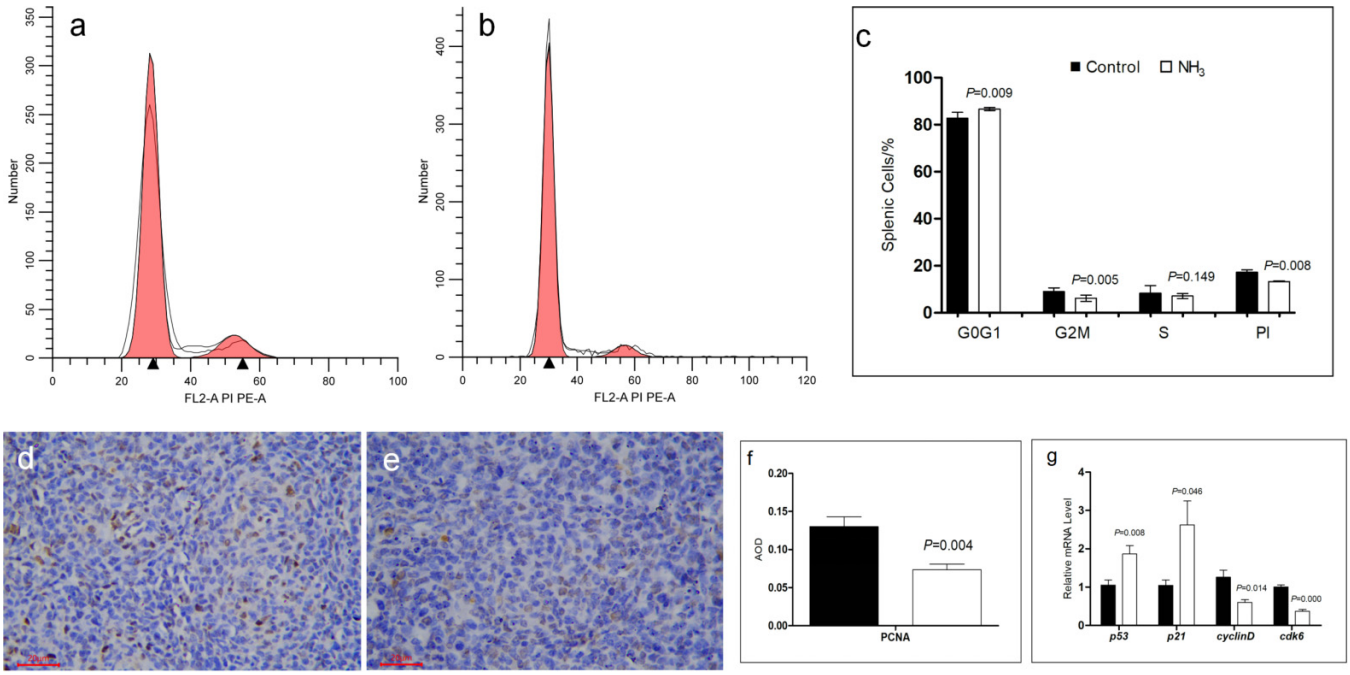
According to the qPCR analysis of relative expressions of cyclin genes, the upregulated expressions of P53 and P21, and downregulated expressions of cyclin D1 and CDK6 were observed in the NH_3 group ($P < 0.05$) compared with the control group (Fig. 3*g*).

Apoptotic percentage and expression of apoptosis regulator

By FCM, the percentage of apoptotic splenocytes in the NH_3 group was significantly higher ($P < 0.01$) than that in the control group (Fig. 4*a*, 4*b*, and 4*g*). 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_3 group (Fig. 4*f*, 4*k*, and 4*r*).

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_3

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_3 challenge) and the NH_3 group (70 ± 5 ppm NH_3) at day 8 of the experiment. Note: Showing the parameters related to the cell cycle. The percentage of G_0G_1 phase cells is higher ($P < 0.05$) and G_2M phase cell distribution and PI are lower ($P < 0.05$) in the NH_3 group than in the control group (*c*). On the histogram from FCM detection, the decrease of G_2M peak height is shown directly in the NH_3 group (*b*) compared with the control group (*a*). By the IHC method, there are fewer PCNA positive cells in the NH_3 group (*e*) than in the control group (*d*), and the OD values of positive materials were lower in the NH_3 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. 4*c*, 4*d*, and 4*h*). 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_3 group were higher ($P < 0.05$) (Fig. 4*e*, 4*f*, and 4*i*).

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. 4*l*–4*q*). 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_3 group compared to the control group. At the same time, the ratio of Bax/Bcl-2 was increased significantly ($P < 0.05$) (Fig. 4*s*).

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_3 group ($P < 0.05$). However, the expressions of Bcl-2 were significantly downregulated in the NH_3 group ($P < 0.05$) (Fig. 4*s*).

Detection of MDA content and T-AOC

As shown in Fig. 4*s*, the contents of MDA were significantly increased ($P < 0.05$), and the T-AOC was decreased ($P = 0.057$) in the NH_3 group compared with the control group.

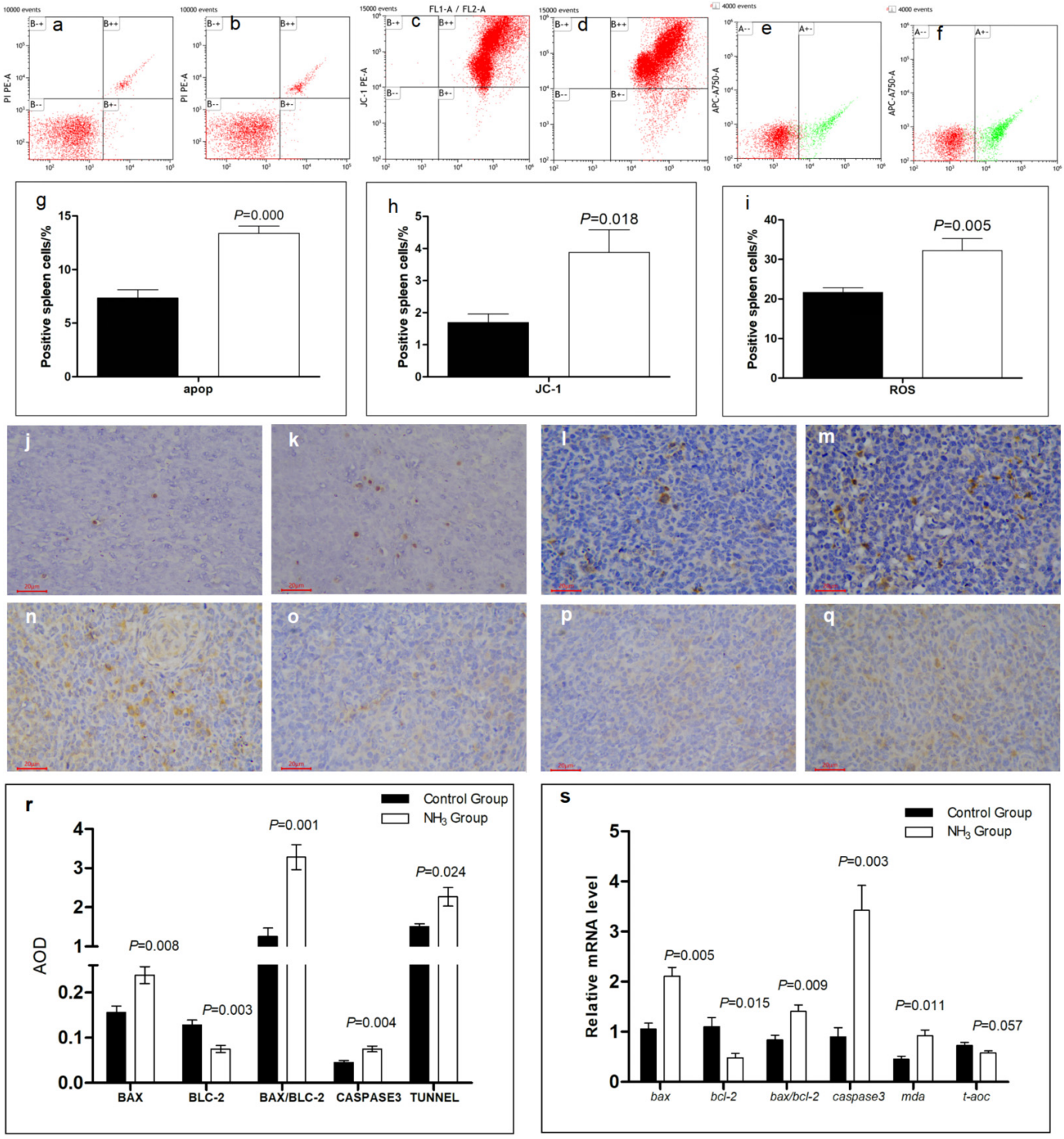
Discussion

According to a review paper, atmospheric NH_3 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_3 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_3 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_3 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_3. The results showed that the development of the spleen could be inhibited by NH_3 exposure, shown by the decrease of relative weight, dysplasia of white pulps, and expansion of mitochondria and ER in lymphocytes.

Cell cycle, including G_0, G_1, S, G_2, 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_3 challenge) and the NH_3 group (70 ± 5 ppm NH_3) 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_1/S phase and the G_2/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_0/G_1 phase) was increased and the percentages of cells during the dividing period (G_2/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_3 group was lower, indicating that NH_3 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_1 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_1 phase to S phase, which could further induce cell cycle blockage at G_0/G_1 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_3 on immunity, it was reported that NH_3 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_3 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 concentration of MDA suggested that the oxidative stress in the spleen could be triggered by NH_3 exposure in broilers. To judge the condition of MMT 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_3. 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_3 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_0/G_1 phase arrest of the cell cycle, and excessive apoptosis by touching off mitochondria-mediated apoptotic pathways. The results suggested that NH_3 exposure could induce spleen lesions in chickens, and its possible mechanisms were cell cycle arrest and apoptosis activation.

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

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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.

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