Ammonia-Induced Energy Metabolism Disorder and Autophagy via AMPK/mTOR/ULK1 Pathway in Chicken Livers

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Research

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Abstract

Background

Ammonia (NH₃) is a well-known environmental pollution gas, threatening human health. NH₃ is also the most harmful gas to poultry for many years. Some studies have found NH₃ can damage eyes, respiratory system, and digestive system. However, molecular mechanism of NH₃ toxicity on chicken livers remains unclear.

Methods

In this study, we selected chicken liver as research object and successfully duplicated NH₃ poisoning model of chickens. The ultrastructure of chicken livers was observed. The activities of ATPases (Na⁺K⁺-ATPase, Mg⁺⁺-ATPase, Ca⁺⁺-ATPase, and Ca⁺⁺Mg⁺⁺-ATPase) and the expression of energy metabolism-related genes (HK1, HK2, PK, PFK, PDHX, CS, LDHA, LDHB, AMPK, SDHA, SDHB, and avUCP) and autophagy-related genes (PI3K, LC3I, LC3II, Beclin1, SQSTM1, mTOR, ULK1, ATG5, ATG12, and ATG13) were measured to explore the effect of NH₃ on energy metabolism and autophagy in chicken livers.

Results

Our results showed that excess NH₃ caused liver tissue damage. Meanwhile, ATPases activities were inhibited during NH₃ treatment. Moreover, we found that NH₃ exposure altered the expression of energy metabolism-related and autophagy-related genes. NH₃-induced compensatory increase of AMPK activated autophagy process through inhibiting mTOR and promoting ULK1. In addition, there was dose-dependent and time-dependent effects on all detected indexes in NH₃-caused chicken liver damage.

Conclusion

Excess NH₃ induced energy metabolism disorder and autophagy via AMPK/mTOR/ULK1 pathway in chicken livers.

Introduction

Ammonia (NH₃), a colorless gas with strong irritant odor and easily soluble in water, can released into the atmosphere through nature and human activities. The plants in forests and grasslands, and the evaporation of oceans and soil all release NH₃ [1]. Man-made sources of NH₃ mainly include farmland fertilizer [2], garbages [3], animal husbandry [4], chemical industry [5], and vehicle exhaust emissions [6]. Excess NH₃ emission in the atmosphere can not only disrupt the global greenhouse balance but also cause air pollution [7]. For example, it can form ammonium salt aerosol with acid gas to participate in PM2.5 formation and contribute to the production of acid rain [8, 9]. It was reported that about 75% of NH₃ emission in European atmosphere came from livestock production [10]. Some researches found that
NH₃ in the poultry house is mainly generated from two aspects: In the process of protein being digested by enzymes in poultry digestive tracts [8] and microbial degradation in chicken manure and bedding [11].

Chickens are sensitive to NH₃. Chickens growing in high concentration of NH₃ for a long time appear tearing and inflammation of cornea and conjunctiva in chicken eyes [12, 13]. In addition, high-concentration NH₃ can damage respiratory system and digestive system of chickens. Two studies showed that excess NH₃ caused immune disorders and inflammatory damage [14], and necro-injury in chicken tracheas [15]. Zhang J et al. found that NH₃ exposure interfered with nutrient absorption in small intestinal mucosae of broilers [16]. NH₃ exposure can also damage immune function in chickens[17–19]. Liver is the largest gland in organism and plays important roles in metabolism, detoxification, and immunity. Schaerdel AD et al. demonstrated that NH₃ can be absorbed into blood through the lungs of rats, resulting in the increase of blood NH₃ [20]. The increase of NH₃ in blood can aggravate hepatic detoxification burden on NH₃ and cause liver NH₃ poisoning. A study found that long-term exposure to high-concentration NH₃ triggered chronic liver injury in broilers [21]. Excess NH₃ also induced hepatic injury via promoting apoptosis in rats [22] and chickens [23]. Therefore, it is of great significance to explore the effect of NH₃ on poultry health, especially on livers.

Energy metabolism is the process of material metabolism, accompanied by the release, transfer, storage, and utilization of energy. During energy metabolism process, adenosine triphosphate (ATP) cannot penetrate cell membrane, and the absorption and utilization of nutrients depend on the electrochemical proton gradient produced by ATPase [24]. ATPases, a group of membrane-binding enzymes, are necessary for normal cell function and help to maintain membrane potential and osmotic balance of cells [25, 26]. Adenosine 5′-monophosphate-activated protein kinase (AMPK), as a whole-body energy sensor, can inhibit energy consumption process and activate energy production process through integrating different signaling pathways to meet energy needs of cells [27]. NH₃ neurotoxicity can induce brain energy metabolism disorder in patients with chronic and acute hepatic encephalopathy [28]. Selenium deficiency inhibited the expression of genes related to energy metabolism and lead to energy metabolism disorder in broiler erythrocytes [29]. Another study showed that excess NH₃ can lead to metabolic disorder in chicken thymuses [30]. Under cell starvation condition, AMPK can trigger autophagy by inhibiting mammalian target of rapamycin (mTOR) and activating unc-51 like autophagy activating kinase 1 (ULK1) to meet cell energy needs [31]. AMPK/mTOR/ULK1 signaling pathway is one of classical pathways connecting energy metabolism and autophagy[31, 32]. Shear stress induced autophagy in vascular smooth muscle cells via activating AMPK/mTOR/ULK1 pathway [33]. Autophagy is a self-degradation process of cellular components through autophagosome-lysosome pathway [34]. Autophagy can serve as a cellular defense mechanism to respond to external harmful stimuli by degrading protein aggregates, damaged organelles, and even pathogens in cells [35]. Autophagy initiation depends on the participation of a series of autophagy-related genes (ATG), such as mTOR, ULK1, Phosphatidylinositol 3-kinase (PI3K), microtubule associated protein 1 light chain 3 (LC3), Beclin1, sequestosome 1 (SQSTM1), ATG5, ATG12, and ATG13. Zhang et al. (2008) studied the changes of autophagy after traumatic brain
injury in rats and found that double membrane structure of autophagy increased, and the protein levels of LC3 and Beclin1 increased in neurons and astrocytes, which indicated that autophagy was activated after brain injury [36]. Harmful substance cadmium exposure increased mRNA and protein expression of Beclin1, LC3I, LC3II, and ATG5, and induced autophagy in chicken kidneys [37]. Another substance NH₃ exposure increased the expression of classical autophagy markers LC3 and Beclin1, and decreased SQSTM1, indicating that excess NH₃ caused autophagy in skeletal muscle of patients with liver cirrhosis [38]. In this study, we successfully established the model of NH₃ poisoning in chickens, observed chicken liver morphology, and measured ATPases (Na⁺K⁺-ATPase, Mg⁺⁺-ATPase, Ca⁺⁺-ATPase, and Ca⁺⁺Mg⁺⁺-ATPase) activities and the expression of energy metabolism-related genes (HK1, HK2, PK, PFK, PDHX, CS, LDHA, LDHB, AMPK, SDHA, SDHB, and avUCP) and autophagy-related genes (PI3K, LC3I, LC3II, Beclin1, SQSTM1, mTOR, ULK1, ATG5, ATG12, and ATG13) to explore molecular mechanism of energy metabolism disorder and autophagy induced by NH₃ in chicken livers.

Materials And Methods

Animals and NH₃ treatment

All procedures used in our experiment were performed in accordance with the requirements of the Northeast Agricultural University's Institutional Animal Care and Use Committee with the approval number SRM-06. All chickens were housed in environmental control cabins at the Laboratory Animal Center, College of Veterinary Medicine, Northeast Agricultural University (Harbin, China). One hundred and eight healthy 1-day-old chickens for fattening were randomly divided into three groups (3 replicates per group and 12 chickens per replicate): the low NH₃ group (≤ 5 mg/m³ during day 1–42), the middle NH₃ group (10 ± 0.5 mg/m³ during day 1–21, 15 ± 0.5 mg/m³ during day 22–42), and the high NH₃ group (20 ± 0.5 mg/m³ during day 1–21, 45 ± 0.5 mg/m³ during day 22–42). A cylinder of compressed anhydrous NH₃ (Dawn Gas Co. Ltd., Harbin, China) and a Photoacoustic Field Gas-Monitor Innova-1412 (Lumasense Technologies, Inc., Santa Clara, CA, USA) were used to produce and control NH₃ concentration in each environmental control cabin. Feed and water were provided ad libitum. On the 14th, 28th, and 42nd days, the chickens were euthanized (200 mg/kg sodium pentobarbital) and livers were collected.

Ultrastructural observation

Liver tissues were cut into about 0.5-1 mm³ tissue blocks. The tissue blocks were fixed in 2.5% (v/v) glutaraldehyde solution, were rinsed with phosphate buffered saline (pH = 7.2), and then were fixed in 1% (v/v) osmium tetroxide. After being dehydrated with gradient ethanol solution, the tissue blocks were impregnated with epoxy resin and were cut into ultrathin sections. Obtained ultrathin sections were stained with uranyl acetate and lead citrate. The observation and record of liver ultrastructure were performed using a transmission electron microscope (GEM-1200ES, Japan Electron Optics Laboratory Co., Ltd, Tokyo, Japan).

ATPase activity detection
The activities of Na\textsuperscript{+}K\textsuperscript{+}-ATPase, Ca\textsuperscript{++}-ATPase, Mg\textsuperscript{++}-ATPase, and Ca\textsuperscript{++}Mg\textsuperscript{++}-ATPase were detected with a kit (Kit number: A016-2) following the manufacturer's instructions (Nanjing Construction Bioengineering Research). All samples were repeatedly detected three times in a single assay using a spectrophotometer (ELX800, BioTek Instruments Inc., Winooski, USA).

**Quantitative Real-time PCR (qRT-PCR)**

mRNA expression of energy metabolism-related and autophagy-related genes was evaluated using qRT-PCR method. Total RNA was isolated from chicken liver tissues with RNAiso Plus (Takara, Japan). Complementary DNA was synthesized in a 20 µL system with FastKing RT Kit (With gDNase) (Kit number: KR116, Tiangen Biotech Co., Ltd., Beijing, China). All primers (Table 1) used in our experiment were synthesized by Invitrogen Biotechnology Co. Ltd. (Shanghai, China). The qRT-PCR was performed in a 20 µL reaction mixture (Roche, Switzerland) with a QuantStudio 3 real-time PCR system (Applied Biosystems, Foster City, CA, USA).
### Table 1
Primer sequences for qRT-PCR

| Gene  | Forward Primer                        | Reverse Primer                        |
|-------|---------------------------------------|---------------------------------------|
|       |                                       |                                       |
| β-actin | 5′-CCGCTCTATGAAGGCTACGC-3′           | 5′-CTCTCGGCTGTTGTTGGA-3′              |
| HK1   | 5′-CTCTCGGAGGACGGCGATG-3′             | 5′-GAAGGCTACAGACACAGACTCG-3′          |
| HK2   | 5′-TGGAGGTGAACGGGAGGTG-3′             | 5′-GCACACAGACACAGACAGAAG-3′           |
| PFK   | 5′-ATCAGTGGAGGTGGCGAAGG-3′            | 5′-CATGTCGGTGGCGAAGAGTC-3′            |
| PK    | 5′-GCTCCACGGTCTCAACAAGAATCC-3′        | 5′-AAGGCACACAGACAGACAGACAC-3′         |
| LDHA  | 5′-TGCCTGTCTGGGAGGAGT-TG-3′           | 5′-GTCCACCACCTGCTTGAACC-3′            |
| LDHB  | 5′-GCAGGTTTCTCAAGCAAGG-3′             | 5′-GGCAGGCAACCTTAACCTCATG-3′          |
| PDHX  | 5′-AGGCTGTGCCTTCTGCTTCAAG-3′          | 5′-TGTCACAGTCGACGAGCACATG-3′          |
| CS    | 5′-CATCCCCACGGCAGACGAGG-3′            | 5′-GTCAGCCACAGCACAGACACCTC-3′         |
| SDHA  | 5′-GAAGAGCAGTGGAGGAAGC-ACAC-3′        | 5′-AGCGAATAGCAGGTGGAACACTTG-3′        |
| SDHB  | 5′-TGGAGGGACTCTATGAGTGCTAC-3′         | 5′-TTGAAGTTGTGCGGAGCTTCC-3′           |
| avUCP | 5′-GCTTCCGCTCCATCCGCATC-3′            | 5′-GCCTGGAAGCAGCATTGAC-3′             |
| AMPK  | 5′-GTTAAGAGAGTAGGGGAGGAAG-3′          | 5′-CGGAAGCGAGTGGCAGATG-3′             |
| PI3K  | 5′-AGAGCCGTGTCCCTTCTTAC-3′            | 5′-TGTCGCGAATAGGCTAGATGAC-3′          |
| mTOR  | 5′-AACCAGTGCCTGGACACATC-3′            | 5′-CATAGGATCGCAGAGCACCAGTAG-3′        |
| SQSTM1| 5′-CCCTCGTTATGGAATCCTCTGCT-3′         | 5′-GAGGCTGTGGTGAATACCTGATGG-3′        |
| Beclin1 | 5′-GACCAGTGGGCACGAGGGGAC-3′         | 5′-CTGGGCTGTACTTCTCGTGCTG-3′          |
| LC3I  | 5′-ATGGCAGAGGTGCAGGGGACTC-3′          | 5′-GGGTGAGTGGAGCAGCATCCAAAC-3′        |
| LC3II | 5′-AGTGAGTGCTAGGAGATGGA-3′            | 5′-AAGCCTTTGGAACGAGAT-3′              |
| ULK1  | 5′-GAGCAAGAAGCACCAGCAGATCC-3′         | 5′-TTTCAGGGCAATCTCCATCACC-3′          |
| ATG5  | 5′-GCCTTCAGTGCCGGTTCCAGTTCC-3′        | 5′-TATCGTCCAAACCAACCATCTCG-3′         |
| ATG12 | 5′-TCCTGAAGGCTGAGGCCCCTCGTGAG-3′      | 5′-TCCCAACTTCTCGTGTAAGATG-3′          |
| ATG13 | 5′-AGGCTCCGAGTTCTCTGACCAC-3′          | 5′-CCCGTAACGCAATCTGTAGTACC-3′         |

### Western blot analysis

Total protein on the 42nd day was extracted with Cell lysis buffer for Western and IP (Beyotime, Shanghai, China, Catalog No. P0013) and Phenylmethanesulfonyl fluoride (Beyotime, Shanghai, China, Catalog No. ST506). Protein concentration was determined with BCA Protein Assay Kit (Kit number:
P0012S, Beyotime, Shanghai, China). The total protein was separated through SDS-polyacrylamide gel electrophoresis and was transferred onto nitrocellulose membranes in Tris–glycine buffer containing 20% methanol. After being blocked with 5% skim milk at 37 °C for 2 h, nitrocellulose membranes were incubated at 4 °C overnight with primary antibodies (β-actin, HK1, HK2, PK, SDHA, SQSTM1, PI3K, ULK1, and ATG12) (produced by the laboratory of Dr. Shiwen Xu, College of Veterinary Medicine, Northeast Agricultural University, Harbin, China), and then were washed three times in phosphate buffered saline with tween 20 (15 min each time), the membranes were incubated with peroxidase-conjugated and horseradish peroxidase-conjugated secondary antibodies. Freshly-made BeyoECL Plus (Beyotime, Shanghai, China, Catalog No. P0018S) was added and finally the membranes were imaged using a chemiluminescence instrument (Amersham Imager 600 RGB, General Electric Company, USA).

Statistical analysis

The data analysis was performed using GraphPad Prism 6.0 (GraphPad Software, Inc., USA). One-way and two-way analyses of variance with Tukey's multiple comparison test were used to analyze statistical significance. Data were given as mean ± standard deviation (SD). Different lowercase letters represented significant differences (P<0.05) among different groups at the same time point, and different uppercase letters represented significant differences (P<0.05) in the same group among different time points.

Results

Ultrastructural changes

The ultrastructure of chicken liver cells was examined using a transmission electron microscope, and ultrastructural changes were shown in Fig. 1. There was no autophagosome and autolysosome in the low NH₃ group (Fig. 1a). Autophagosomes and autolysosomes enclosing a few organelles were observed in the middle NH₃ group (Fig. 1b). The ultrastructure of chicken liver cells in the high NH₃ group (Fig. 1c1-c2) showed typical autophagy characteristics such as more autophagosomes and autolysosomes enclosing a large number of organelles.

ATPase activity

The activities of Na⁺K⁺-ATPase, Mg⁺⁺-ATPase, Ca⁺⁺-ATPase, and Ca⁺⁺Mg⁺⁺-ATPase were presented in Fig. 2. On the 14th, 28th, and 42nd days, the activities of above four ATPases decreased significantly (P<0.05) with the increase of NH₃ concentration at all three time points.

For the low NH₃ group, there was no significant difference (P>0.05) in the activities of above four ATPases among three time points. For the middle NH₃ group and the high NH₃ group, the four ATPase activities decreased significantly (P<0.05) with the increase of NH₃ treatment time except for Mg⁺⁺-ATPase activity in the middle NH₃ group between the 14th day and the 28th day.
mRNA and protein expression of energy metabolism-related genes

To further study the effect of NH$_3$ on energy metabolism in chicken livers, we measured mRNA expression of HK1, HK2, PFK, PK, PDHX, CS, LDHA, LDHB, AMPK, SDHA, SDHB, and avUCP on days 14, 28, and 42; and protein expression of HK1, HK2, PK, and SDHA, on day 42 as shown in Fig. 3.

At three time points, AMPK mRNA expression in the low NH$_3$ group is the lowest significantly ($P<0.05$), and in the high NH$_3$ group was the highest significantly ($P<0.05$). However, contrary to what happened in AMPK, mRNA expression of all other genes in the low NH$_3$ group is the most highest significantly ($P<0.05$), and mRNA expression of all other genes in the high NH$_3$ group was the lowest significantly ($P<0.05$) except there was no significant difference ($P>0.05$) in HK2, PKM, and SDHB between the low NH$_3$ group and the middle NH$_3$ group on the 14th day.

For the low NH$_3$ group, there was no significant differences ($P>0.05$) in mRNA expression of all above genes among different time points. For the middle and the high NH$_3$ groups, AMPK mRNA expression increased significantly ($P<0.05$) with NH$_3$ treatment duration. Instead, mRNA expression of all other genes decreased significantly ($P<0.05$) with NH$_3$ treatment duration except there were no significant difference ($P>0.05$) in PFK and LDHA in the middle NH$_3$ group between the 14th day and the 28th day.

In addition, we found that protein expression of the four genes decreased significantly ($P<0.05$) with NH$_3$ treatment concentration.

mRNA and protein expression of autophagy-related genes

In our experiment, qRT-PCR and Western blot analysis were used to detect mRNA expression and protein expression of autophagy-related genes, respectively. The results were shown in Fig. 4. On the 14th, 28th, and 42nd days, compared with the low NH$_3$ group, mRNA expression of LC3I (b), LC3II (c), Beclin1 (d), ULK1 (g), ATG5 (h), ATG12 (i), and ATG13 (j) in the middle NH$_3$ group and the high NH$_3$ group increased significantly ($P<0.05$), and mRNA expression of above genes was the highest significantly ($P<0.05$) in the high NH$_3$ group. However, mRNA expression of PI3K (a), SQSTM1 (e), and mTOR (f) in the middle NH$_3$ group and the high NH$_3$ group was significantly lower than those in the low NH$_3$ group ($P<0.05$), and mRNA expression of above genes was the lowest significantly in the high NH$_3$ group ($P<0.05$). Protein expression of PI3K and SQSTM1 decreased significantly, and protein expression of ULK1 and ATG12 increased significantly with the increase of NH$_3$ concentration on day 42 ($P<0.05$).

There was no significant difference in mRNA expression of detected autophagy-related genes among different time points in the low NH$_3$ group ($P>0.05$). Regarding the middle NH$_3$ group and the high NH$_3$ group, mRNA expression of LC3I, LC3II, Beclin1, ULK1, ATG5, ATG12, and ATG13 increased significantly with the increase of NH$_3$ exposure time ($P<0.05$) except for there was no significant difference in Beclin1.
between on the 14th day and on the 28th day ($P > 0.05$). mRNA expression increased with the increase of NH$_3$ exposure time, but there was no significant difference between the 14th day and the 28th day ($P > 0.05$). Besides, mRNA expression of PI3K, SQSTM1, and mTOR decreased significantly with the increase of NH$_3$ exposure time ($P < 0.05$).

**Discussion**

Cells can show a series of morphological changes to response to stress after being stimulated by harmful substances. For example, hydrogen sulfide exposure can lead to nuclear membrane contraction, chromatin aggregation and marginalization, mitochondrial cristae swelling and vacuolation [39]. Previous studies have shown that NH$_3$ toxicity can cause pathological changes in cell morphology and lead to tissue damage. A study found that the morphology of human hepatic stellate cells changed dramatically, with spindle shaped fibroblast phenotype and signs of abnormal lumen of lysosome after NH$_3$ treatment [40]. There are many autophagic bodies with double vesicles in skeletal muscle cells of mice treated with high NH$_3$ [38]. Similar to the above studies, in our experiment, high-concentration NH$_3$ resulted in tissue damage in chicken livers. The ultrastructure showed that a large number of mitochondrial cristae broke, and autophagy bodies and autophagy lysosomes wrapped with a large number of organelles appeared, indicating that the toxicity of NH$_3$ resulted in the destruction of mitochondrial structure and obvious autophagy damage.

Some researchers found that harmful stimulation can damage mitochondrial function, destroy energy metabolism balance, and lead to the disorder of energy metabolism. Chi Q et al. found that harmful gas H$_2$S exposure led to the damage of chicken spleen mitochondrial structure, decreased ATPase activity and the expression of energy metabolism related genes (HK2, PK, PDHX, SDHB and avUCP), which indicating that energy metabolism disorder occurred in chicken spleens [41]. Mitochondrial dysfunction and energy metabolism disorder were related to NH$_3$ toxicity [42]. Another report demonstrated that H$_2$S can also reduce the expression of AMPK, HK1, PFK, LDHA and LDHB, leading to the disorder of energy metabolism in chicken livers [43]. NH$_3$ toxicity inhibited SDH activity, leading to mitochondrial membrane potential collapse, mitochondrial swelling, and ATP depletion, which confirmed that mitochondrial dysfunction and energy metabolism disorder occurred in mice livers and brains under NH$_3$ toxicity [44]. In order to explore the effect of NH$_3$ on energy metabolism of chicken livers, we measured the expression of energy metabolism-related genes (HK1, HK2, PK, PFK, PDHX, CS, LDHA, LDHB, SDHA, SDHB, and avUCP) and found that NH$_3$ inhibited above energy metabolism-related genes and caused energy metabolism disorder in chicken livers. ATPase can catalyze the process of ATP decomposition and release energy to supply cells to complete various biological reactions, which plays a key role in the energy conversion and utilization of biological cells. Accordingly, it is very important to maintain ATPase activity in energy metabolism process. Cao C et al. reported that selenium deficiency inhibited the activities of Na$^+$/K$^+$-ATPase, Ca$^{++}$-ATPase, and Ca$^{++}$/Mg$^{++}$-ATPase in chicken arteries and veins [45]. Lead exposure reduced the activities of Na$^+$/K$^+$-ATPase, Mg$^{++}$-ATPase, and Ca$^{++}$-ATPase in chicken kidney tissues [46]. Hence, in
our present experiment, we further determined the activities of \( \text{Na}^+\text{K}^-\text{ATPase} \), \( \text{Mg}^{++}\text{ATPase} \), \( \text{Ca}^{++}\text{ATPase} \), and \( \text{Ca}^{++}\text{Mg}^{++}\text{ATPase} \), and found that high-concentration \( \text{NH}_3 \) decreased the activities of above four \( \text{ATPases} \), meaning that excess \( \text{NH}_3 \) inhibits energy metabolism in chicken livers. In addition, we found that the decrease of the expression of energy metabolism-related genes and \( \text{ATPases} \) activities was dose-dependent and time-dependent. Our above results suggested that \( \text{NH}_3 \) exposure destroyed energy metabolism balance, which may lead to energy deficiency. AMPK plays an important role in signal pathway of energy and substrate metabolism, and is a central energy sensor to maintain metabolic homeostasis \cite{47}. Interestingly, AMPK mRNA and protein expression increased significantly under \( \text{NH}_3 \) exposure. This may be due to that upon activation, AMPK can inhibit energy required for cellular activities and meanwhile stimulate catabolic pathways \cite{48}. The decrease of energy level in liver tissues activated AMPK. AMPK compensatively satisfied energy demand through inhibiting energy consumption process, activating energy production process, and even activating autophagy process. Hence, the compensatory increase of AMPK level further reflected energy metabolism disorder induced by \( \text{NH}_3 \) in chicken livers.

Autophagy is closely related to energy metabolism disorder. Under energy deficiency state, AMPK can directly phosphorylate ULK1, thus further promoting autophagy \cite{49}. Phosphoserine phosphatase can inhibit autophagy in hepatocellular carcinoma cell via the AMPK/mTOR/ULK1 signaling pathway \cite{50}. ULK1 complex is a bridge between upstream energy receptor AMPK/mTOR and downstream autophagy \cite{49}. Wang S et al. demonstrated that cadmium exposure led to energy metabolism disorder, meanwhile the expression of autophagy-related genes LC3, ATG5, and Beclin1 increased significantly, and mTOR expression decreased, suggesting the occurrence of energy metabolism disorder and autophagy in chicken ovariess \cite{51}. Cadmium exposure inhibited mTOR expression by inducing AMPK expression, and increased the expression of LC3I, LC3II and Beclin1, which triggered BNIP3 dependent autophagy pathway \cite{52}. Under normal conditions, PI3K can phosphorylate mTOR to maintain the rich activity of mTOR in tissues, thus inhibiting ULK1 and ATG13 complex and preventing them from starting autophagy \cite{53}. Autophagy signal can inhibit mTOR and activate ULK1. Once activated, ULK1 can phosphorylate ATG13 and induce Beclin1 activation and other autophagy activating molecules to recruit autophagy proteins to participate in the formation of autophagy bodies \cite{54}. Therefore, AMPK/mTOR/ULK1 pathway plays an important role in regulating autophagy process. ATG5, a key protein involved in membrane extension of phagocytes in autophagic vesicles, forms a complex with ATG12 and locates on the outer membrane of autophagy, which is crucial for autophagic precursors formation \cite{55}. ATG5/ATG12 complex can promote the binding of LC3I to autophagic membrane. The membrane binding LC3II is an important marker molecule of autophagy, which increases with the increase of autophagic membrane \cite{34}. SQSTM1, as an autophagic substrate marker, can connect LC3 and ubiquitination substrates, and then be integrated into autophagy and degraded in autophagy lysosomes \cite{56}. Deng X et al. reported that PM2.5 increased the level of LC3, ATG5 and Beclin1 in a time- and concentration-dependent manner, suggesting that PM2.5 induced autophagy in A549 cells \cite{57}. Puerarin reduced autophagic bodies formation in hippocampal CA1 area after cerebral ischemia-reperfusion injury, decreased the expression of Beclin1, the ratio of LC3II/LC3I, AMPK and ULK1, and increased SQSTM1 expression, indicating that
puerarin alleviated autophagy through inhibiting the activation of AMPK/mTOR/ULK1 signaling pathway [58]. NH₃ toxicity induced autophagy by inhibiting mTOR expression and increasing expression of LC3I, LC3II, Beclin1, and ATG5, which confirmed that NH₃ toxicity induced cardiac autophagy in chickens [59]. In this study, we measured the expression of autophagy-related genes (PI3K, LC3I, LC3II, Beclin1, SQSTM1, mTOR, ULK1, ATG5, ATG12 and ATG13) in chicken livers. The results were consistent with above mechanisms and researches. NH₃ exposure triggered AMPK/mTOR/ULK1 signaling pathway through inhibiting mTOR and activating ULK1 and induced autophagy, and energy metabolism disorder mediated autophagy caused by NH₃ in chicken livers.

**Conclusion**

Taken together, our results demonstrated that NH₃ exposure damaged liver tissue, inhibited ATPases activities, and altered the expression of energy metabolism-related and autophagy-related genes. There was dose-dependent and time-dependent effect on all detected indexes in NH₃-caused chicken liver damage. Excess NH₃ induced energy metabolism disorder and autophagy. Energy metabolism disorder mediated NH₃-induced autophagy by triggering AMPK/mTOR/ULK1 pathway (Fig. 5).

**Abbreviations**

Ammonia
NH₃; Adenosine triphosphate:ATP; Adenosine 5’-monophosphate-activated protein kinase:AMPK; Mammalian target of rapamycin:mTOR; unc-51 like autophagy activating kinase 1:ULK1; autophagy-related gene:ATG; Phosphatidylinositol 3-kinase:PI3K; Microtubule associated protein 1 light chain 3:LC3; Sequestosome 1:SQSTM1; Autophagosome:Au; Autolysosome:Ai

**Declarations**

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**Authors’ contributions**

Zhuo Li and Zhiying Miao designed the study, performed experiments, collected the experimental data, and drafted the manuscript. Linlin Ding analyzed the experimental data and assisted in laboratory work. Jun Bao and Xiaohua Teng designed the study, and critically reviewed the manuscript. All the authors read and approved the final manuscript.
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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on request.

Ethics approval

All procedures used in our experiment were performed in accordance with the requirements of the Northeast Agricultural University’s Institutional Animal Care and Use Committee with the approval number SRM-06.

Consent for publication

Not applicable.

Competing interests

All authors read and approved the final manuscript. The authors declare that there are no conflicts of interest.

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**Figures**
Figure 1

Ultrastructural changes in chicken liver cells. a: the low NH3 group (× 10,000). b: the middle NH3 group (× 20,000). c1 and c2: the high NH3 group (× 20,000). Au: autophagosome. Ai: autolysosome.
Figure 2

The activities of Na⁺K⁺-ATPase, Mg⁺⁺-ATPase, Ca⁺⁺-ATPase, and Ca⁺⁺Mg⁺⁺-ATPase in chicken livers. Each value represented mean ± SD (n = 12). Different lowercase letters represented significant differences (P < 0.05) among different groups at the same time point, and different uppercase letters represented significant differences (P < 0.05) in the same group among different time points.
Figure 3

mRNA and protein expression of energy metabolism-related genes in chicken livers. Each value represented mean ± SD (n = 12). Different lowercase letters represented significant differences (P < 0.05) among different groups at the same time point, and different uppercase letters represented significant differences (P < 0.05) in the same group among different time points.
Figure 4

The expression of autophagy-related genes in chicken livers. Each value represented mean ± SD (n = 12). Different lowercase letters represented significant differences (P < 0.05) among different groups at the same time point, and different uppercase letters represented significant differences (P < 0.05) in the same group among different time points.
Figure 5

NH3 exposure induced oxidative stress-mediated energy metabolism disorder and autophagy via APMK/mTOR/ULK1 signaling pathway in chicken livers.