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Copper toxicity in the liver of broiler chicken: insights from metabolomics and AMPK-mTOR mediated autophagy perspective

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ABSTRACT Exposure to copper (**Cu**) has been associated with metabolic disorders in animals and humans, but the underlying mechanism remains unclear. One-day-old broiler chickens, numbering a total of 192, were nourished with dietary intakes that contained varying concentrations of Cu, specifically 11, 110, 220, and 330 mg/kg of Cu, for a period extending over a duration of 7 wk. As a result of the study, Cu exposure resulted in vacuolization, fragmentation of mitochondria cristae, and the increase of autophagosomes in hepatocytes. Metabolomics analysis illustrated that Cu caused a total of 59 different metabolites in liver, predominantly associated with the glycerophospholipid metabolic pathway, leading to metabolic disruption. Moreover, high-Cu diet markedly reduced the levels of AMPKα1, p-AMPKα1, mTOR, and p-mTOR and enhanced the expression levels of the autophagy-related factors (Atg5, Dynein, Beclin1, and LC3-II). Overall, Cu exposure caused chicken liver injury and resulted in disturbed metabolic processes and mediated autophagy primarily through the AMPK-mTOR axis.

Key words: Copper, metabolomics, AMPK-mTOR, autophagy, liver

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INTRODUCTION

Copper (**Cu**), a vital constituent for organisms, facilitates animal growth through their diet and assumes a pivotal function in myriad physiological phenomena, including but not limited to hematopoiesis, digestion, growth, reproduction, and immunity (Zhong et al., 2024). Previous study has revealed that the inclusion of Cu at a concentration of 30 mg/kg in animal feed could facilitate growth and development (Li et al., 2021). The liver serves as the primary reservoir and processing center for Cu, facilitating its storage and metabolic functions. Cu enters hepatocytes to form Cu-containing histidine trimethyl inner salt firstly, and then transfer to Cu-containing enzymes, which present an indispensable part in biological processes such as mitochondrial respiration, antioxidant defense and iron metabolism (Altarelli et al., 2019). Nevertheless, a study has discovered that consuming overabundant amounts of Cu (150 mg/kg) could harm the animals and inhibit their growth (Attia et al., 2012). With the widespread use of Cu-containing feeds, pesticides, and fertilizers, large amounts of Cu are released into the soil and water bodies. Subsequently, the Cu accumulation in the soil and water presents a formidable hazard to the well-being of both human and animal populations via biological aggregation (Seiler and Berendonk, 2012). Excessive Cu intake could cause Cu superload in hepatocytes, leading to partial organelle injury which eventually leads to metabolic disorders and impairment (de Romana et al., 2011). Our antecedent investigations have demonstrated that diets rich in Cu have the potential to trigger oxidative stress leading to apoptosis in the hepatic tissues of poultry (Yang et al., 2021). However, the specific mechanism between metabolic disorders and cell death in organisms has not yet been entirely explored.

Autophagy, an intricate process of lysosomal degradation that widely exists in eukaryotic cells and essential for the maintenance of normal body functions (Denton et al., 2015). It can eliminate damaged organelles and harmful components, as well as regulate intracellular homeostasis under stress conditions (Parzych and Klionsky, 2014). Nevertheless, an overabundance of autophagy may result in cellular impairment and apoptosis,

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consequently jeopardizing holistic well-being. Excessive initiation of autophagy could be triggered by heavy metal toxicity, oxidative stress, malnutrition, and inflammatory processes (Kim and Lee, 2014). Our previous study has substantiated the capacity of Cu to elicit oxidative stress and autophagy in boar testes (Li et al., 2021). Indeed, autophagy functions as a metabolic process that governs energy homeostasis. It is orchestrated by a network of autophagy-related genes and a diverse array of signaling pathways, among which mTOR stands out. The regulation of autophagy has been demonstrated to be intricately linked to alterations in both the internal and external environments, as well as to metabolic shifts (Hou et al., 2022). However, the exploration of the intricate relationship between liver metabolism and autophagy remains relatively limited, necessitating further research and investigation to unravel its complexities.

The metabolism and pathways of cell death are considered crucial determinants of cell fate. Disruption of the cellular homeostasis can lead to alterations in metabolic products due to impaired cellular function. mTOR complex Ⅰ acts the optimal regulator of autophagy is which perceives the metabolic state of cells and is a principal regulator of bioenergetic pathways and cell growth (Zhang et al., 2019). There are many upstream pathways of mTOR signaling molecules. The mTOR signaling cascade encompasses a myriad of upstream pathways, including the noteworthy involvement of AMPK, functioning as a pivotal metabolic regulator. AMPK assumes the position of a metabolic switch that skillfully orchestrates the facilitation of energy-producing pathways involved, including fatty acid oxidation, mitochondrial biogenesis, and glucose uptake (Trefts and Shaw, 2021). In addition, it effectively curtails processes of energy consumption including protein and lipid synthesis (Zhang et al., 2023). Accumulating evidence supports a link between the AMPK/mTOR signaling pathway and autophagy (Ploumi et al., 2022). The liver serves as the epicenter of bodily metabolism, orchestrating the equilibrium of energy substances within the bloodstream to facilitate optimal functioning of the organism. The purpose of this study was to explore the hepatotoxicity of Cu in broiler chickens, and focus on alterations of metabolic processes and the autophagy regulation, which provided valuable new perspectives on the mechanism of Cu-induced hepatic injury from the viewpoints of metabolomics and autophagy.

MATERIALS AND METHODS

Animals and Treatment

All procedures of the animal experiments were ratified by the Ethics Committee of South China Agricultural University (NO. 2017A087). The experimental animals were 192 one-day-old commercial white-feather broiler chickens were fed the diet with 11 mg/kg, 110 mg/kg, 220 mg/kg, and 330 mg/kg Cu for 7 wk. Cu sulfate ($\mathbf{CuSO_4}$) (Analytical Reagent, 99.7%) purchased from Xilong Scientifific (China) was used as the source of Cu. During the experiment, all broiler chickens were kept in coops and free to eat and drink. The liver is taken out at 1, 3, 5 and 7 wk after intravenous injection of sodium pentobarbital anesthesia.

Histopathological Observation

In the 7th wk, liver tissue was immobilized with 4% paraformaldehyde (Sigma, USA), dewatered with ethanol, paraffin-embedded, and sliced. Then, the segments underwent staining with hematoxylin and eosin (H&E) and were scrutinized for histological alterations under illumination from a light microscope.

Ultrastructural Observation

Reference the steps in the previous research (Jiang et al., 2024), the livers were fixed, rinsed, dehydrated, and fixed-embedded in resin in the 7th wk. The specimen was sliced using an ultramicrotome and then subjected to staining with uranyl acetate and lead acetate. Subsequently, the outcomes were observed using a transmission electron microscope manufactured by Hitachi in Japan.

Metabonomics

In the 7th wk, metabolites were extracted from frozen tissues of the control group and the 330 mg/kg Cu group for metabolomic sequencing. The experimental methods and data analysis in this section reference previous research conducted in the laboratory (Liao et al., 2021). The Human Metabolome Database (HMDB; http://www.hmdb.ca/) serves as a resource for contrasting metabolites, while MetaboAnalyst 4.0 (http://www.metaboanalyst.ca/) and KEGG (http://www.genome.jp/kegg/) are employed for metabolite pathway analysis.

Flow Cytometry

During the 7 wk, hepatocytes from broiler chickens were isolated, and the MDC signals were discerned employing a specialized kit. The excitation wavelength stood at 488 nm, while the emission wavelengths were at 525 nm. Subsequently, visual documentation was conducted.

Immunohistochemistry

Fix the sample in a 4% solution of paraformaldehyde. Incubate with primary antibody against BECN1 (purchased from Zhongshan Biotech Co., Ltd.) at 4℃ for 18 h. Subsequent to incubation, the specimen underwent exposure to a secondary antibody conjugated with horseradish peroxidase (**HRP**) at ambient temperature for a duration of 60 min. Finally, treat the sample with a solution of 3,3′-diaminobenzidine (purchased from Nanjing Jiancheng Bioengineering Institute), observe the Beclin1

positive signal under a Leica microscope (purchased from Germany), and capture corresponding images.

Immunofluorescence

Referring to previous study (Zhang et al., 2023), After conventional processing of the tissue slices, they were incubated at 4℃ for 18 h with LC3Ⅱ antibody (Abcam, Britain). Subsequently, they were nurtured at ambient temperature for one hour with TRITC-labeled secondary antibody. Next, a 3-minute DAPI counterstaining was performed using DAPI (Solarbio, China). Lastly, an anti-fluorescence quencher was added to the tissue slices, and they were observed using a laser scanning confocal microscope (Leica, Germany).

Real-Time Quantitative Polymerase Chain Reaction

Following the previous study (Shi et al., 2024), RNA was extracted from liver tissue at wk 1, 3, 5, and 7, and converted into complementary DNA (**cDNA**) following established protocols employing the Reverse Transcription Kit from Takara, Japan. Subsequently, quantitative polymerase chain reaction (**qPCR**) was executed utilizing the Light Cycler 480 System by Roche. The relative messenger RNA (**mRNA**) expression levels were evaluated employing the $2^{-\Delta\Delta CT}$ technique (Sun et al., 2024). The primer sequences are delineated in Table 1.

Western Blot Analysis

Extract total protein from the liver of broiler chickens in wk 7, following the procedures of the previous Western Blot experiment (Liao et al., 2021). Protein content was determined and quantified using the BCA Protein Assay Kit, and then electrophoresed on an SDS polyacrylamide gel and transferred to a PVDF membrane. After rapid closure for 20 min, the PVDF membrane was incubated with antibodies at 4℃. The primary antibodies used were AMPKα1 (1:500, Santa Cruz), p-AMPKα1 (1:1000, Bioss, China), mTOR (1:1000, ImmunoWay), p-mTOR (1:1000, Affinity), Atg5 (1:1000, Abcam) and Beclin1 (1:1000, Sino Biological lnc., China). Use GAPDH (1:5000, Bioss, China) as a control. Incubation was then performed with peroxidase-coupled antibodies against rabbit or mouse IgG. Lastly, Detect protein bands using the BioRad Chemi Doc XRS system, and analyze them using Image J software.

Table 1. Primer sequences for target genes.

	Primer sequence
AMPKα1	F: ATCTGTCTCGCCCTCATCCT
	R: CCACTTCGCTCTTCTTACACCTT
mTOR	F: GAAGAGCTGATTCGGGTAG
	R: ACCATTCTTGTGCCTCCATT
Dynein	F: CGGCTTGACCTATGGAATCT
	R: CATCACTGCGAGGAACTGC
ATG5	F: AGAGATGTGTGGTTTGGACGC
	R: GCCGAGGAAGGGCTGTATT
Beclin1	F: CGTATGGCAACCACTCGTATT
	R: TTATTGTCCCAGAAGAACCTCAG
GAPDH	F: AACCAGCCAAGTATGATGAT
	R: ACCATTGAAGTCACAGGAG

Statistical Analysis

At least 3 repetitions of each experiment were performed and the data were presented as mean ± standard deviation (**SD**). Least significant difference (**LSD**) and one-way analysis of variance (**ANOVA**) tests were utilized to assess the variation among groups. $P < 0.05$ indicated that the data were markedly divergent.

RESULTS

Effects of Cu on Histomorphology and Ultrastructure in the Liver of Broiler Chicken

The results of liver section in the 7th wk were illustrated in Figure 1A. With the elevated dose of Cu treatment, liver tissue showed worsening vacuolization in various groups, with the most pronounced pathological damage in the 330 mg/kg Cu group. The ultrastructural morphology is shown in Figure 1B. Autophagosomes were visible in the 110 mg/kg Cu group (red arrows), and the mitochondrial ridge structure was blurred, fragmented, and disordered in the 220 mg/kg Cu group (green arrows). The hepatocyte structure of the 330 mg/kg Cu group was severely damaged, with disappearance of the nuclear membrane, mitochondrial swelling and vacuolization, fractured and blurred mitochondrial ridges, and the appearance of numerous engulfing bubbles. The degree of liver cell damage has a significant dose-effect relationship with the Cu content in broiler chicken feed.

Effects of Cu on the Change of Metabolic in the Liver of Broiler Chicken

To explore the impact of Cu exposure on liver metabolism, metabolomic sequencing was applied to analyze the liver metabolism after Cu exposure. As shown in Figure 2A, the metabolism of the 330 mg/kg Cu group was remarkably altered, with 59 different metabolites including both positive and negative. There was an additional 14 metabolites and a reduction of 45 metabolites by Cu exposure. As shown in Figure 2B, Cu exposure influenced 18 metabolic pathways in the liver by enrichment pathway analysis, of which the 4 most essential pathways were glycerophospholipid metabolism, glutathione metabolism, pentose and glucuronate interconversions, and biotin metabolism. We additionally discovered that the expression levels of hepatic LysoPC (18:2(9Z,12Z)), uridine diphosphate glucose, PC (14:0/20:2(11Z,14Z)), Dolichol phosphate, LPA (0:0/18:0), 3-O-Sulfogalactosylceramide were enhanced in the Cu-treated group. In addition, the expression levels of S-Adenosylmethioninamine, Biocytetin, Glutathionate, D-Xylose, 5′-Methylthioadenosine,

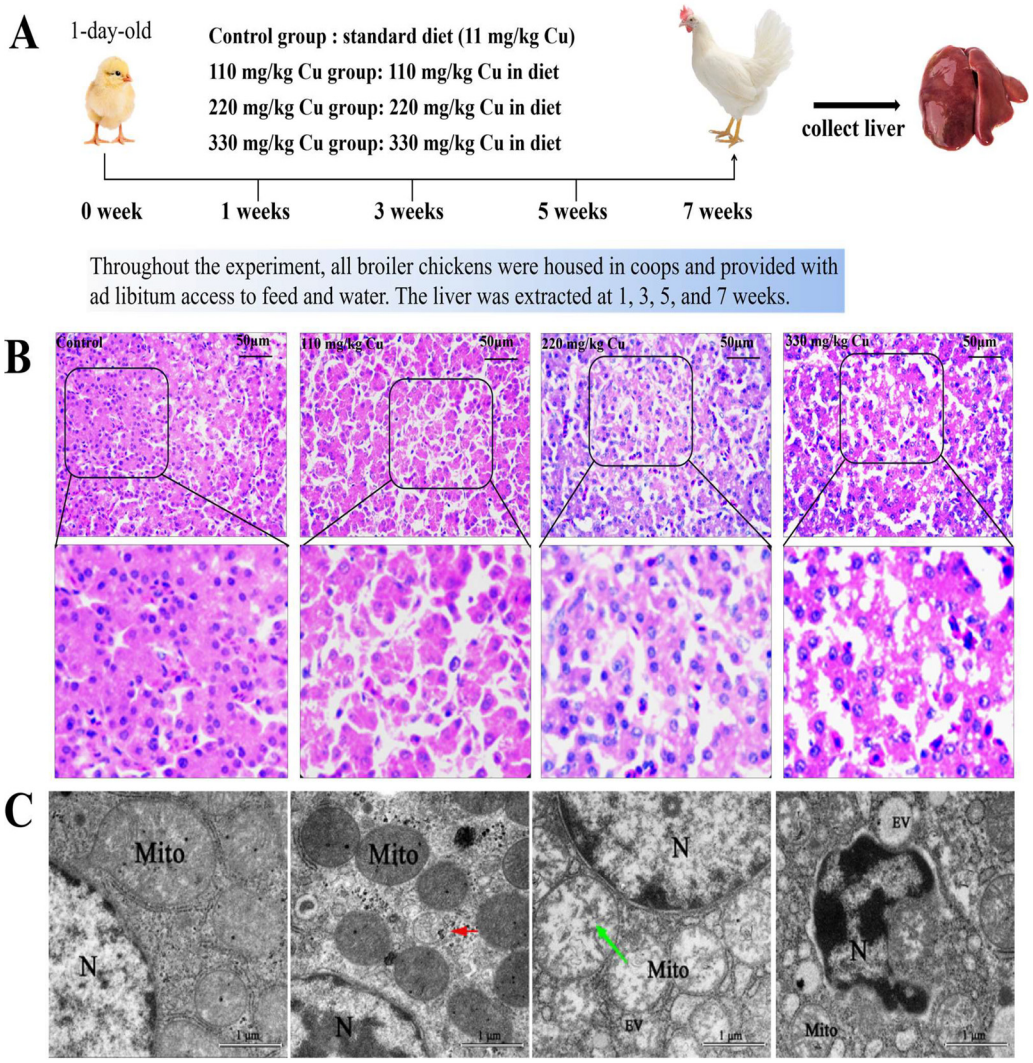

Figure 1. Construction of Cu exposure model and its effect on histomorphology and ultrastructure of broiler chicken liver. (A) Schematic diagram of model construction of Cu exposure. (B) The histological changes in liver. The scale bar was 50 μm. (C) The ultrastructural changes in liver. The red arrow indicates autophagosome structure, and the green arrow indicates mitochondrial ridge disorder. "N" means cell nucleus. "Mito" means mitochondria. "EV" means engulfing vesicle. The scale bar is 1 μm.

PE (22:6(4Z,7Z,10Z,13Z,16Z,19Z)/P-18:1(9Z)) were decreased (Figure 2C).

Effects of Cu on the mRNA Levels of Autophagy-Related Genes in the Liver of Broiler Chicken

The mRNA levels of AMPKα1, mTOR, Beclin1, ATG5 and Dynein in liver of broiler chicken at wk 1, 3, 5, and 7 were shown in Figures 3A−3E. In Figure 3A, the expression level of AMPKα1 mRNA was elevated only in the 330 mg/kg Cu group at the 3 wk, and dramatically decreased in all groups containing different concentrations of Cu at the 7 wk. The expression level of mTOR was notably elevated in the 220 mg/kg Cu group at 3 wk, but decreased in the 220 and 330 mg/kg Cu groups at 5 wk and 7 wk, and evidently decline in the 110 mg/kg Cu group at 7 wk (Figure 3B). Beclin1 was dramatically raised in the 330 mg /kg Cu group at wk 3 and in the 110, 220, and 330 mg Cu /kg groups at wk 7 (Fig. 3C). The expression level of ATG5 was obviously increased in the 330 mg/kg Cu group at wk 3 and the 220 and 330 mg/kg Cu groups at wk 5 and 7 (Figure 3D). Dynein was remarkedly higher in the 330 mg/kg Cu group than that in the control group at wk 3, the 110 mg/kg Cu group at wk 5, and the 110 and 220 mg/kg Cu groups at wk 7 (Figure 3E).

Effects of Cu on the Expression Levels of Autophagy-Related Proteins in the Liver of Broiler Chicken

The concentrations of autophagy-associated proteins in the hepatic tissue of broiler chickens in each experimental cohort at the 7th wk were delineated in Figure 4. As can be seen from the figure, the protein expression levels of AMPKα1 and mTOR exhibited a conspicuous decline in both the 220 and 330 mg/kg Cu group. In addition, the p-AMPKα1 and p-mTOR protein expression levels were markedly decreased in different

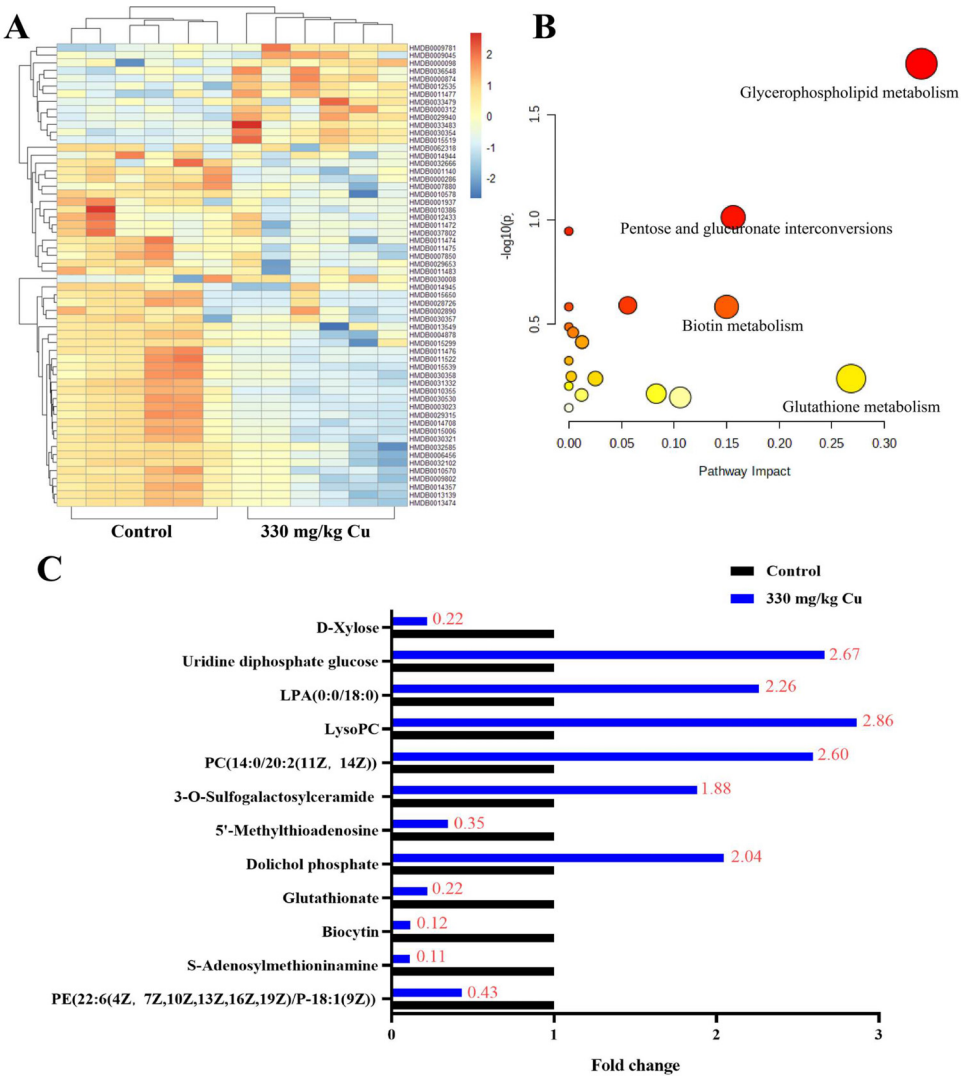

Figure 2. Differential metabolites and metabolic pathways in Cu-exposed broiler chicken livers. (A) Heat map showing differential metabolites in the liver. (B) Pathway analysis of metabolites in the liver. (C) Fold changes of different metabolites related to glycerophospholipid metabolism, glutathione metabolism, pentose and glucuronate interconversions, and biotin metabolism.

concentrations of Cu-treated groups. After feeding with Cu diet, the Beclin1 protein expression levels in each group did not exhibit marked differences from those observed, however, they initially rose and subsequently fell as the dietary Cu content increased. The ATG5 protein level in liver cells of broilers in all groups was increased, and the difference was significant in 330 mg/kg Cu group (Figure 4D).

Effects of Different Concentrations of Cu-Containing Diets on Autophagy Level in the Liver of Broiler Chicken

To further investigate the level of liver autophagy after Cu exposure, we extracted chicken hepatocytes to detect MDC signaling using flow cytometry. The outcomes displayed a remarkable increase in MDC production in the 110 and 220 mg/kg Cu group (Figures 5A and 5B). The immunohistochemical expression levels of Beclin1 in hepatocytes of broiler chicks at wk 7 are illustrated in Figure 5C. The brown positive particles in the 110 mg/kg Cu group were primarily expressed in the cytoplasm of hepatocytes with low expression, and the number of brown positive particles was elevated in the 220 and 330 mg/kg Cu group. The LC3II immunofluorescence expression level was remarkably raised in the 220 and 330 mg/kg Cu group (Figure 5D).

DISCUSSION

Cu is crucial for life processes, but it could lead to poisoning of the organism in excess (Gurnari and Rogers, 2021). Currently, Cu pollution in the environment has prompted significant apprehension due to its extensive utilization in both agricultural practices and industrial manufacturing. It has been recognized for a long time that hepatotoxicity has a direct causal relationship with Cu exposure (Zhong et al., 2023). Observation of the ultrastructure in one study revealed that rat liver mitochondria showed different degrees of damage after Cu

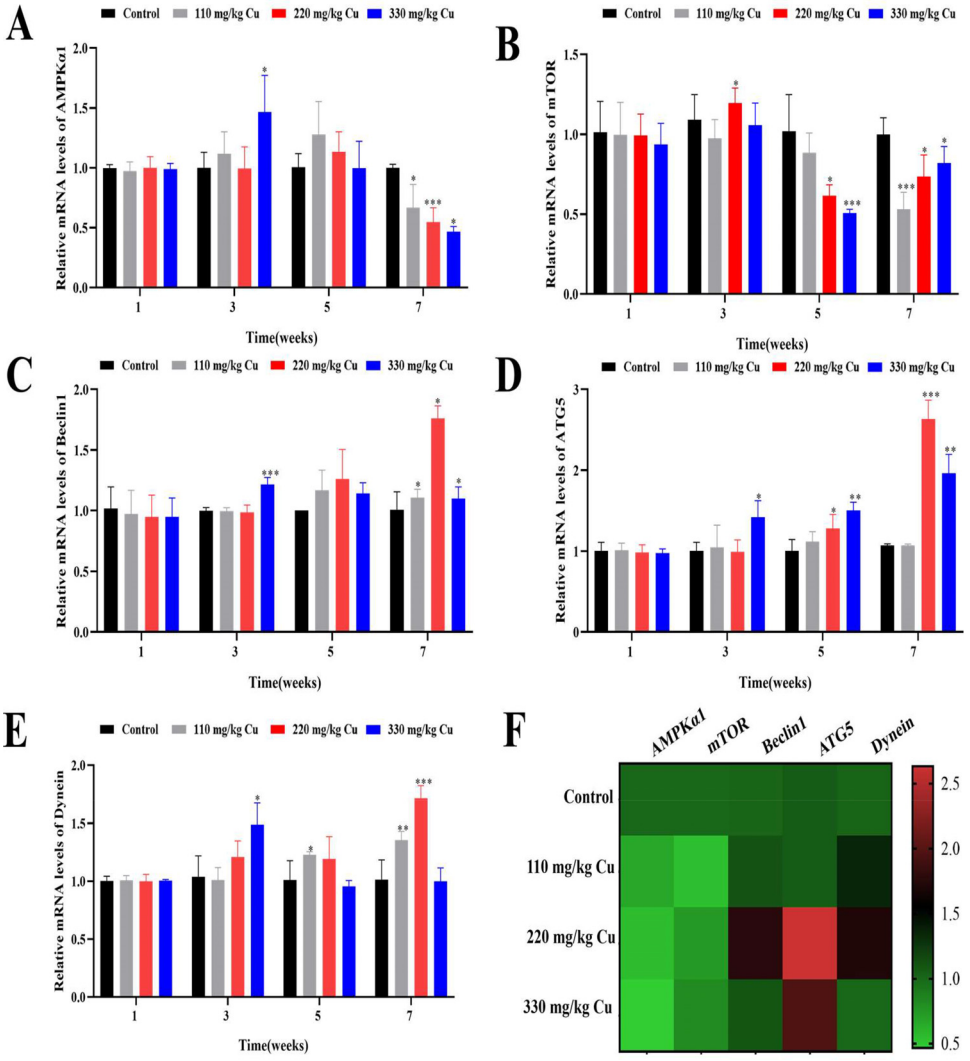

Figure 3. Effects of Cu exposure on autophagy-related genes in broiler chicken liver. (A-E) mRNA expression levels of autophagy-related genes at wk 1, 3, 5 and 7. (F) Heat map of the expression levels of autophagy-related mRNAs at 7 wk. ($^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$).

exposure (Yu et al., 2021). In the present study, it was observed that Cu exposure resulted in vacuolar degeneration of broiler liver tissue and mitochondrial vacuolization, ridge swelling and ridge breakage.

To delve into the impact of Cu exposure on the hepatic metabolism of broiler chicken, PCA, PLS-DA, heatmap, and hierarchical clustering were employed to analyze differential metabolites and metabolic pathways. Nanoplastics have been identified to alter metabolic pathways including tricarboxylic acid (**TCA**)-related cycles, purine metabolism and glutathione (**GSH**) metabolism in human liver L02 cells (Lin et al., 2022). Our findings indicated significant differences in the liver metabolome of broiler chickens exposed to 330 mg/kg Cu at 7th wk. A grand total of 59 distinct metabolites were discerned, exhibiting precise modulation within the realms of lipid metabolism, glucuronic acid metabolism and other pathways. Lipids and their derivatives including linoleic acid, α-linoleic acid, and arachidonic acid are primary cell membrane constructs (Harayama and Riezman, 2018), and serve a dominant function in modulating various signal transduction pathways including cell proliferation (Storck et al., 2018) and autophagy (Soto-Avellaneda and Morrison, 2020). Low level of glutathione has been noted to correlate with elevated concentrations of reactive oxygen species, resulting in oxidative harm to proteins, lipids, and DNA (Escribano et al., 2015). Disturbances in glutathione metabolism may result in increased oxidative stress, consequently interfering with glycerophospholipid metabolism and impacting overall energy metabolic processes in the body (Wang et al., 2021). In addition, sphingolipid metabolism is a complicated system composed of associated metabolites, and the alteration of sphingolipid metabolism is also associated with the alteration of glycerophospholipid metabolism and glutathione metabolism due to the significantly increased level of 3′ -O-sulfogalactosyllactam induced by Cu (Blaber et al., 2017). Moreover, fatty acids as an indispensable energy substance can cause the destruction of mitochondrial energy metabolism, leading to autophagy (Riffelmacher et al., 2017). In this investigation, the incorporation of 330 mg/kg of Cu into the dietary regimen demonstrated the potential to augment the

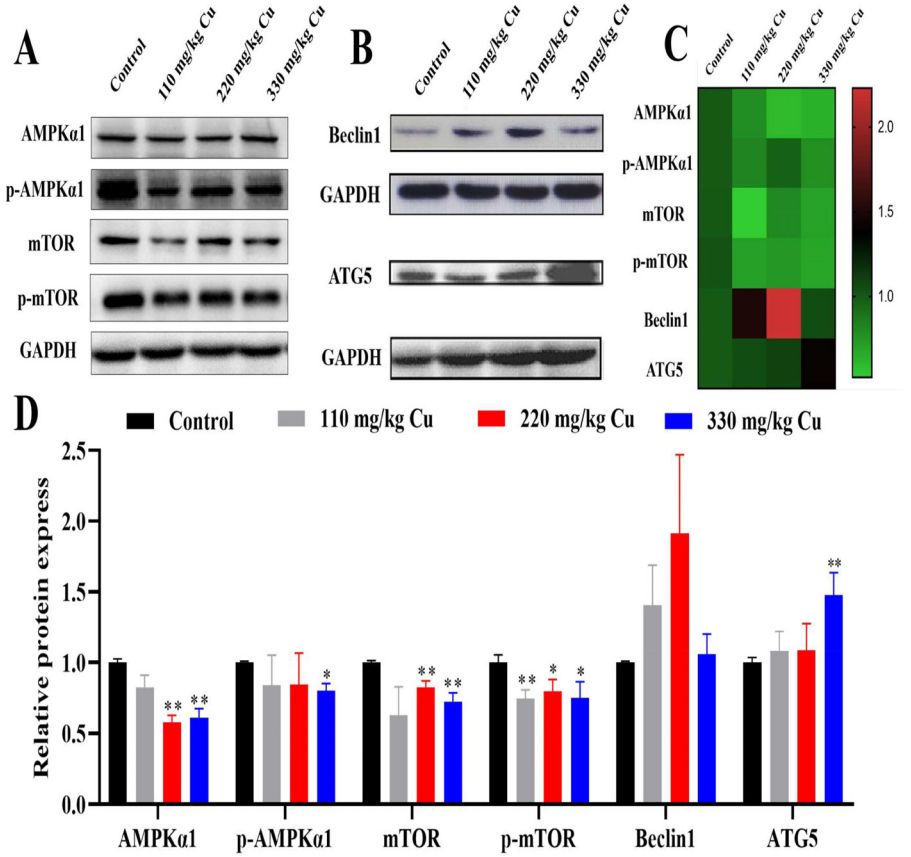

Figure 4. Effects of Cu exposure on autophagy-related proteins in broiler chicken livers. (A-B) Western blot detection bands of autophagy-related proteins at 7 wk. (C) Heat map of autophagy-related protein expression levels at 7 wk. (D) Gray value analysis results of autophagy-related proteins at 7 wk.

concentration of glucose uridine diphosphate, consequently modulating the dynamics of glucuronic acid metabolism. Ultimately, Cu exposure influences the glycerophospholipid metabolism pathway and the glutathione metabolism pathway via the glucuronide pathway and the tricarboxylic acid cycle. Our data showed that Cu exposure resulted in remarkable changes in lipid metabolic pathways, including fatty acid elongation, glycerophospholipid metabolism and fatty acid degradation. Previous investigations have indicated that autophagy consistently coincided with alterations in glycerophospholipid composition (encompassing phosphatidylcholine, lysophospholipid, phosphoethanolamine, lysophosphatidic acid, etc.) situated on the exterior of cellular membranes or organelles, a phenomenon intricately intertwined with the metabolic cascade of glycerophospholipids (Calzada et al., 2016; Shen et al., 2018). Fascinatingly, in the present study, the differences in glycerophospholipid metabolic pathway production were closely related to the regulation of various signaling cascades of autophagy.

AMPK, a critical energy metabolism kinase, acts a pivotal effect in regulating biological metabolism (Tamargo-Gomez and Marino, 2018). It has been proposed in studies that AMPK function may be somewhat impaired in the presence of metabolic dysfunction, as in the case of heavy metal exposures and cardiovascular diseases (Steinberg and Hardie, 2023; Tinkov et al., 2023). Inactivation or weakening of AMPK may lead to a reduction in the energy production pathway, an enhancement of the energy expenditure pathway, and disruption of lipid and glucose metabolism in the body (Spaulding and Yan, 2022). A metabolomics study revealing interactions between AMPK and energy metabolism, mice receive an external stimulus that alters a variety of metabolites and disrupts AMPK-glycogen binding in vivo (Belhaj et al., 2022). It is widely acknowledged that the AMPK/mTOR signaling pathway stands as one of the pivotal regulatory pathways governing autophagy. It was identified that nickel exposure could regulate the AMPK/AKT-mTOR pathway through oxidative stress and induced autophagy in TCMK-1 cells (Yin et al., 2024). mTOR functions as a pivotal threonine/serine protein kinase that serves as a crucial regulator in the process of autophagy (Wang and Zhang, 2019). A decrease in mTOR expression activates the process of autophagy, stimulating its occurrence through mechanisms such as the acceleration of autophagosome generation and fusion (Huang et al., 2023). Researches have demonstrated that the induction of autophagy in the testes of ducks by heavy metals is associated with the AMPK/mTOR signaling pathway (Pu et al., 2023). Equally, the reduction of AMPK and mTOR expression and the activation of autophagy was discovered after Cu exposure, in the present experiments. At the onset of autophagy, Beclin1 recruits many

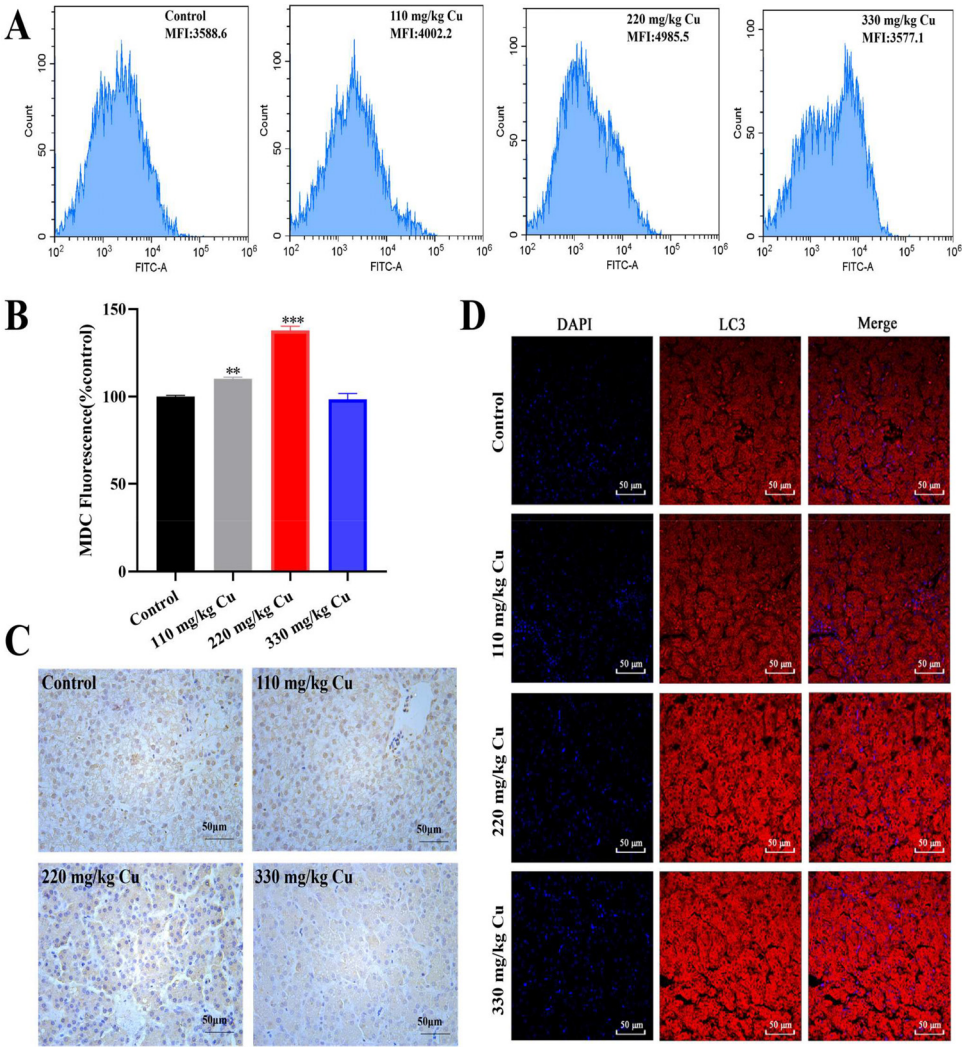

Figure 5. Effects of different concentrations of Cu-containing diets on autophagy in broiler chicken liver. (A) MDC fluorescence levels of autophagy in hepatocytes were detected by flow cytometry. (B) MDC mean fluorescence intensity histogram (percent control). (C) Beclin1 was stained by immunohistochemical detection, scale bar: 50 μm. (D) LC3-II was stained by immunofluorescence, scale bar: 50 μm.

autophagic proteins together to form autophagosomes (Shi et al., 2019), and ATG5 can establish complex associations with various autophagy-associated proteins, which subsequently attach to autophagosome membranes in order to accelerate the recruitment of LC3 to autophagosomes (Arakawa et al., 2017). The recent studies revealed that dynein exerts a pivotal position in the transport and fusion of autophagosome contents to lysosomes, thus hindering the clearance of aggregation-prone proteins (Lie and Nixon, 2019). Cadmium exposure was reported to induce autophagy by activating the expression of P62, ATG5, and Beclin-1 in chicken hepatocytes (Li et al., 2023). In this research, we discovered that Cu exposure activated the mRNA levels of autophagy-related factors, and autophagy signals were detected by flow cytometry. Meanwhile, immunohistochemistry and immunofluorescence positive expression of Beclin1 and LC3 were significantly elevated in the liver. All the above results showed that Cu exposure induced hepatic metabolic disorders and inhibited the AMPK/mTOR signaling pathway, thereby inducing hepatic autophagy in broiler chickens.

CONCLUSIONS

To summarize, Cu exposure resulted in chicken liver damage and altered multiple metabolites and metabolic pathways, and induced autophagy in the chicken liver via the suppression of the AMPK/mTOR signaling pathway. This study enriches the toxicological mechanisms of Cu from the perspective of metabolomics and autophagy.

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All authors have read the manuscript and agreed to submit it in its current form for consideration for publication in the journal.

DISCLOSURES

The authors declare no conflicts of interest.

REFERENCES

Altarelli, M., N. Ben-Hamouda, A. Schneider, and M. M. Berger. 2019. Copper deficiency: causes, manifestations, and treatment. Nutr Clin Pract 34:504–513.

Arakawa, S., S. Honda, H. Yamaguchi, and S. Shimizu. 2017. Molecular mechanisms and physiological roles of Atg5/Atg7-independent alternative autophagy. Proc. Jpn. Acad. Ser. B Phys. Biol. Sci. 93:378–385.

Attia, Y. A., E. M. Qota, H. S. Zeweil, F. Bovera, A. A. Abd, and M. D. Sahledom. 2012. Effect of different dietary concentrations of inorganic and organic copper on growth performance and lipid metabolism of White Pekin male ducks. Br. Poult. Sci. 53:77–88.

Belhaj, M. R., N. G. Lawler, J. A. Hawley, D. I. Broadhurst, N. J. Hoffman, and S. N. Reinke. 2022. Metabolomics reveals mouse plasma metabolite responses to acute exercise and effects of disrupting AMPK-glycogen interactions. Front. Mol. Biosci. 9:957549.

Blaber, E. A., M. J. Pecaut, and K. R. Jonscher. 2017. Spaceflight activates autophagy programs and the proteasome in mouse. Liver. Int. J. Mol. Sci. 18:2062.

Calzada, E., O. Onguka, and S. M. Claypool. 2016. Phosphatidylethanolamine metabolism in health and disease. Int. Rev. Cell Mol. Biol. 321:29–88.

de Romana, D. L., M. Olivares, R. Uauy, and M. Araya. 2011. Risks and benefits of copper in light of new insights of copper homeostasis. J. Trace Elem. Med. Biol. 25:3–13.

Denton, D., T. Xu, and S. Kumar. 2015. Autophagy as a pro-death pathway. Immunol. Cell Biol. 93:35–42.

Escribano, A., M. Amor, S. Pastor, S. Castillo, F. Sanz, P. Codoner-Franch, and F. Dasi. 2015. Decreased glutathione and low catalase activity contribute to oxidative stress in children with alpha-1 antitrypsin deficiency. Thorax 70:82–83.

Gurnari, C., and H. J. Rogers. 2021. Copper deficiency. N. Engl. J. Med. 385:640.

Harayama, T., and H. Riezman. 2018. Understanding the diversity of membrane lipid composition. Nat. Rev. Mol. Cell Biol. 19:281–296.

Hou, L. S., Y. W. Zhang, H. Li, W. Wang, M. L. Huan, S. Y. Zhou, and B. L. Zhang. 2022. The regulatory role and mechanism of autophagy in energy metabolism-related hepatic fibrosis. Pharmacol. Ther. 234:108117.

Huang, J., Z. Chen, Z. Wu, X. Xie, S. Liu, W. Kong, and J. Zhou. 2023. Geniposide stimulates autophagy by activating the GLP-1R/AMPK/mTOR signaling in osteoarthritis chondrocytes. Biomed. Pharmacother. 167:115595.

Jiang, L., F. Yang, H. Liao, W. Chen, X. Dai, C. Peng, Z. Li, H. Wang, T. Zhang, and H. Cao. 2024. Molybdenum and cadmium cause blood-testis barrier dysfunction through ROS-mediated NLRP3 inflammasome activation in sheep. Sci. Total Environ. 906:167267.

Kim, K. H., and M. S. Lee. 2014. Autophagy—a key player in cellular and body metabolism. Nat. Rev. Endocrinol. 10:322–337.

Li, F., L. Liu, X. Chen, B. Zhang, and F. Li. 2021. Dietary copper supplementation increases growth performance by increasing feed intake, digestibility, and antioxidant Activity in Rex Rabbits. Biol. Trace Elem. Res. 199:4614–4623.

Li, N., B. J. Yi, M. Saleem, X. N. Li, and J. L. Li. 2023. Autophagy protects against Cd-induced cell damage in primary chicken hepatocytes via mitigation of oxidative stress and endoplasmic reticulum stress. Ecotoxicol. Environ. Saf. 259:115056.

Liao, J., F. Yang, Y. Bai, W. Yu, N. Qiao, Q. Han, H. Zhang, J. Guo, L. Hu, Y. Li, J. Pan, and Z. Tang. 2021. Metabolomics analysis reveals the effects of copper on mitochondria-mediated apoptosis in kidney of broiler chicken (Gallus gallus). J. Inorg. Biochem. 224:111581.

Lie, P., and R. A. Nixon. 2019. Lysosome trafficking and signaling in health and neurodegenerative diseases. Neurobiol. Dis. 122:94–105.

Lin, S., H. Zhang, C. Wang, X. L. Su, Y. Song, P. Wu, Z. Yang, M. H. Wong, Z. Cai, and C. Zheng. 2022. Metabolomics reveal nanoplastic-induced mitochondrial damage in human liver and lung cells. Environ. Sci. Technol. 56:12483–12493.

Parzych, K. R., and D. J. Klionsky. 2014. An overview of autophagy: morphology, mechanism, and regulation. Antioxid. Redox Signal. 20:460–473.

Ploumi, C., M. E. Papandreou, and N. Tavernarakis. 2022. The complex interplay between autophagy and cell death pathways. Biochem. J. 479:75–90.

Pu, W., X. Chu, H. Guo, G. Huang, T. Cui, B. Huang, X. Dai, and C. Zhang. 2023. The activated ATM/AMPK/mTOR axis promotes autophagy in response to oxidative stress-mediated DNA damage co-induced by molybdenum and cadmium in duck testes. Environ. Pollut. 316:120574.

Riffelmacher, T., A. Clarke, F. C. Richter, A. Stranks, S. Pandey, S. Danielli, P. Hublitz, Z. Yu, E. Johnson, T. Schwerd, J. McCullagh, H. Uhlig, S. Jacobsen, and A. K. Simon. 2017. Autophagy-dependent generation of free fatty acids is critical for normal neutrophil differentiation. Immunity 47:466–480.

Seiler, C., and T. U. Berendonk. 2012. Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. Front. Microbiol. 3:399.

Shen, S., L. Yang, L. Li, Y. Bai, and H. Liu. 2018. Lipid metabolism in mouse embryonic fibroblast cells in response to autophagy induced by nutrient stress. Anal. Chim. Acta 1037:75–86.

Shi, B., M. Ma, Y. Zheng, Y. Pan, and X. Lin. 2019. mTOR and Beclin1: Two key autophagy-related molecules and their roles in myocardial ischemia/reperfusion injury. J. Cell Physiol. 234:12562–12568.

Shi, X., T. Xu, M. Gao, Y. Bi, J. Wang, Y. Yin, and S. Xu. 2024. Combined exposure of emamectin benzoate and microplastics induces tight junction disorder, immune disorder and inflammation in carp midgut via lysosome/ROS/ferroptosis pathway. Water Res 257:121660.

Soto-Avellaneda, A., and B. E. Morrison. 2020. Signaling and other functions of lipids in autophagy: a review. Lipids Health Dis 19:214.

Spaulding, H. R., and Z. Yan. 2022. AMPK and the adaptation to exercise. Annu. Rev. Physiol. 84:209–227.

Steinberg, G. R., and D. G. Hardie. 2023. New insights into activation and function of the AMPK. Nat. Rev. Mol. Cell. Biol. 24:255–272.

Storck, E. M., C. Ozbalci, and U. S. Eggert. 2018. Lipid cell biology: a focus on lipids in cell dvision. Annu. Rev. Biochem. 87:839–869.

Sun W., Lei Y., Jiang Z., Wang K., Liu H. and Xu T., BPA and low-Se exacerbate apoptosis and mitophagy in chicken pancreatic cells by regulating the PTEN/PI3K/AKT/mTOR pathway [e-pub ahead of print]. *J Adv Res*, 10.1016/j.jare.2024.01.029, accessed July, 2024.

Tamargo-Gomez, I., and G. Marino. 2018. AMPK: regulation of metabolic dynamics in the context of autophagy. Int J Mol Sci 19.

Tinkov, A. A., M. Aschner, A. Santamaria, A. R. Bogdanov, Y. Tizabi, M. B. Virgolini, J. C. Zhou, and A. V. Skalny. 2023. Dissecting the role of cadmium, lead, arsenic, and mercury in non-alcoholic fatty liver disease and non-alcoholic steatohepatitis. Environ Res 238:117134.

Trefts, E., and R. J. Shaw. 2021. AMPK: restoring metabolic homeostasis over space and time. Mol Cell 81:3677–3690.

Wang, S., K. S. Tan, H. Beng, F. Liu, J. Huang, Y. Kuai, R. Zhang, and W. Tan. 2021. Protective effect of isosteviol sodium against LPS-induced multiple organ injury by regulating of glycerophospholipid metabolism and reducing macrophage-driven inflammation. Pharmacol. Res. 172:105781.

Wang, Y., and H. Zhang. 2019. Regulation of autophagy by mTOR signaling pathway. Adv. Exp. Med. Biol. 1206:67–83.

Yang, F., J. Liao, W. Yu, N. Qiao, J. Guo, Q. Han, Y. Li, L. Hu, J. Pan, and Z. Tang. 2021. Exposure to copper induces mitochondria-mediated apoptosis by inhibiting mitophagy and the PINK1/

parkin pathway in chicken (Gallus gallus) livers. J. Hazard Mater. 408:124888.

Yin, H., C. Wang, H. Guo, X. Li, and J. Liu. 2024. The mechanism of nickel-induced autophagy and its role in nephrotoxicity. Ecotoxicol. Environ. Saf. 273:116150.

Yu, W., J. Liao, F. Yang, H. Zhang, X. Chang, Y. Yang, R. M. Bilal, G. Wei, W. Liang, J. Guo, and Z. Tang. 2021. Chronic tribasic copper chloride exposure induces rat liver damage by disrupting the mitophagy and apoptosis pathways. Ecotoxicol. Environ. Saf. 212:111968.

Zhang, H. L., Y. M. Zhu, and X. Y. Zhou. 2019. Coordination of autophagy and other cellular activities. Adv. Exp. Med. Biol. 1206:697–727.

Zhang, T., F. Yang, X. Dai, H. Liao, H. Wang, C. Peng, Z. Liu, Z. Li, J. Shan, and H. Cao. 2023. Role of Caveolin-1 on the molybdenum and cadmium exposure induces pulmonary ferroptosis and fibrosis in the sheep. Environ. Pollut. 334:122207.

Zhong, G., L. Li, Y. Li, F. Ma, J. Liao, Y. Li, H. Zhang, J. Pan, L. Hu, and Z. Tang. 2023. Cuproptosis is involved in copper-induced hepatotoxicity in chickens. Sci. Total Environ. 866:161458.

Zhong, G., Y. Li, F. Ma, Y. Huo, J. Liao, Q. Han, L. Hu, and Z. Tang. 2024. Copper exposure induced chicken hepatotoxicity: involvement of ferroptosis mediated by lipid peroxidation, ferritinophagy, and inhibition of FSP1-CoQ10 and Nrf2/SLC7A11/GPX4axis. Biol. Trace Elem. Res. 202:1711–1721.