Research Note: Taraxasterol alleviates aflatoxin B1-induced oxidative stress in chicken primary hepatocytes

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ABSTRACT Aflatoxin B1 (**AFB1**) is the most toxic subtype of aflatoxin in feed. Poultry is sensitive to AFB1, and the liver is the main target organ of AFB1. Our previous studies have shown that taraxasterol isolated from the traditional Chinese medicinal herb *Taraxacum* has protective effects against immune-mediated and alcoholic-induced liver injuries. This study aimed to investigate whether taraxasterol has the protective effect and its mechanism against AFB1-induced injury in chicken primary hepatocytes in vitro. The chicken primary hepatocytes were induced with AFB1 (0.05 μg/mL), and treated with taraxasterol (5, 10, and 20 μg/mL). The results showed that taraxasterol increased superoxide dismutase (**SOD**) and glutathione (**GSH**) activity and decreased malondialdehyde (**MDA**) and reactive oxygen species (**ROS**) production in AFB1-induced hepatocytes. Moreover, taraxasterol up-regulated the mRNA and protein expression of antioxidant-related factors heme oxygenase-1 (**HO-1**), NADPH quinone oxidoreductase 1 (**NQO1**) and nuclear factor erythroid E2-related factor 2 (**Nrf2**), while down-regulated the expression of oxidant-related factor Kelch-like ECH-associated protein 1 (**Keap1**) in Nrf2/Keap1 signaling pathway. In addition, taraxasterol effectively reduced AFB1-induced hepatocyte autophagy and inhibited the mRNA expression of autophagy-related genes Beclin-2, LC3-I, LC3-II, and ATG-5. Taraxasterol also inhibited AFB1-induced hepatocyte apoptosis and decreased the mRNA expression of apoptosis-related genes Caspase3 and Caspase9. These findings indicates taraxasterol alleviates oxidative stress in AFB1-induced chicken hepatocytes by activating Nrf2/Keap1 signaling pathway, and regulating the cell autophagy and apoptosis.

Key words: taraxasterol, aflatoxin B1, chicken primary hepatocytes, oxidative stress

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INTRODUCTION

Aflatoxin is a secondary metabolite produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin widely exists in food and feed, which seriously threatens the health of humans and animals. Aflatoxin contamination is a global problem (Peles et al., 2019). In particular, aflatoxin B1 (**AFB1**) is the most toxic subtype of aflatoxin in feed and poses a serious impact on the health of livestock and poultry, resulting in huge economic losses (Peles et al., 2019). Poultry, especially broiler chickens, are sensitive to AFB1 (Wang et al., 2018). AFB1 in contaminated feed causes organ damage, reduces production performance and feed utilization, inhibits immune function, enhances susceptibility to disease, increases morbidity and mortality, and affects meat quality (Zhao et al., 2021). Moreover, AFB1 accumulated in meat may cause chronic poisoning and even cancer, which seriously endangers the public health and safety (Rushing and Selim, 2019).

The liver is the main target organ of AFB1. AFB1 has a special affinity to the liver and induces oxidative stress in the liver during the metabolic process, and AFB1-induced oxidative stress is the important cause of liver injury (Li et al., 2021). Reactive oxygen species (**ROS**) produced by oxidative stress can trigger hepatocyte apoptosis and autophagy by activating related signaling pathways (Li et al., 2021). Therefore, it is of great significance to understand the process and mechanism of AFB1-induced liver injury for drug screening and prevention.

Taraxacum is a perennial herb with the functions of clearing away heat and detoxification, eliminating swelling and dispersing mass, diuresis and relieving lymph. *Taraxacum* has long been widely used in traditional

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oriental food and medicine because of the significant free radical scavenging, antibacterial, antioxidant, anti-inflammatory, anti-tumor, anti-atherosclerosis and hepatoprotective effects. Taraxasterol is a pentacyclic triterpenoid compound isolated from *Taraxacum*. In recent years, we have carried out a series of studies on the pharmacological effects of taraxasterol such as anti-inflammatory, immune enhancement and antioxidation. Our studies have also shown that taraxasterol has significant protective effects against alcoholic-induced and immune-mediated hepatic injuries (Xu et al., 2018; Sang et al., 2019). However, it is unclear whether taraxasterol has a protective effect against AFB1-induced hepatic injury and potential modes of action. In this study, we explored the effect and its molecular mechanism of taraxasterol by establishing a model of AFB1-induced chicken primary hepatocyte injury in vitro. It lays a foundation for the development of taraxasterol as a potential drug or feed additive preventing and treating AFB1-induced hepatic injury in veterinary clinics.

MATERIALS AND METHODS

Drugs and Reagents

Taraxasterol (purity ≥99.5%) was obtained from Chengdu Fenruisi Biotechnology Co., Ltd. (Chengdu, Sichuan, China). AFB1 was purchased from Qingdao Pribolab Biological Engineering Co., Ltd. (Qingdao, Shandong, China). Fetal bovine serum (**FBS**), methyl thiazolyl tetrazolium (**MTT**), glycogen periodic acid schiff (**PAS**) staining kit and penicillin-streptomycin were purchased from Beijing Solarbio Technology Co., Ltd (Beijing, China). DMEM medium was purchased from Gibco (Australia). Superoxide dismutase (**SOD**), glutathione (**GSH**), malondialdehyde (**MDA**), reactive oxygen species (**ROS**) reagent kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Bicinchoninic acid (**BCA**) kit was purchased from Beyotime Biotech. (Shanghai, China). Real-time fluorescence quantitative PCR kit was purchased from Takara (Osaka, Japan). Primary rabbit antibodies against heme oxygenase-1 (**HO-1**), NADPH quinone oxidoreductase 1 (**NQO1**), nuclear factor erythroid E2-related factor 2 **(Nrf2**), ECH-associated protein 1 (**Keap1**), β-actin and goat anti-rabbit HRP-IgG were purchased from Cell Signaling Technology Inc. (Danvers, MA). CYTO-ID autophagy detection kit was purchased from Enzo Biochem (NY). Annexin V-FITC apoptosis detection kit was purchased from Invitrogen (Carlsbad, CA).

Isolation, Identification and Culture of Chicken Primary Hepatocytes

Chicken primary hepatocytes were extracted from 9 to 14-day-old specific pathogen free chicken embryos (Chinese Academy of Agricultural Sciences, Beijing, China). The isolated cells were identified by glycogen PAS staining. The hepatocytes were cultured in DMEM medium containing 10% FBS at 37°C in a 5% CO_2 cell incubator.

Determination for the Used Doses of AFB1 and Taraxasterol

The chicken primary hepatocytes were incubated in a 96-well plate for overnight. The cells were treated with 0-0.8 μg/mL of AFB1 for 20 h, 20 μL MTT (5 mg/mL) was added and continually cultured for 4 h, 150 μL of DMSO was added and shook, OD value at 490 nm was measured and the cell viability was calculated. On the other hand, the cells were incubated and treated with 0-0.2 μg/mL of AFB1 for 24 h, the supernatant of cells was collected, OD value at 510 nm was measured, and ALT and AST activities were calculated. Similarly, the hepatocytes were incubated and treated with 0 to 100 μg/mL of taraxasterol for 20 h, MTT was added and cultured for 4 h, DMSO was added and shook, OD value at 490 nm was measured and the cell viability was calculated.

Assay for Oxidation Indices

The chicken primary hepatocytes at a density of 5×10^5 cells/mL were incubated for overnight. The experiment was divided into normal group, AFB1 group, taraxasterol (5, 10, and 20 μg/mL, respectively) groups. The cells in taraxasterol groups were induced with 0.05 μg/mL AFB1 and treated with 5, 10, 20 μg/mL of taraxasterol for 24 h, respectively; the cells in AFB1 group were only induced with 0.05 μg/mL AFB1; the cells in normal group were not treated with AFB1 and taraxasterol. After 24 h, the supernatant of hepatocytes was collected and the content of SOD, GSH, MDA, and ROS was determined by using reagent kits according to the manufacturer's instructions.

Quantitative Real-Time PCR

The cell culture and drug treatment were described as the above. The total RNA was extracted with RNAiso Plus kit. The purity and concentration of RNA were determined by detecting Abs260/Abs280 with a spectrophotometer. The cDNA was synthesized from 1 μg of total RNA by RNA reverse transcription kit. The primers were designed by software Primer Premier 6.0 and synthesized by Invitrogen (Shanghai, China). The primers were as follows: Nrf2: forward, GATGTCACCCT GCCCTTAGA and reverse, TCGTTCCATTTGTTCC TTCTG; Keap1: forward, CATCGGCATCGCCAAC TT and reverse: TGAAGAACTCCTCCTGCTTGGA; HO-1: forward, TCATTGGCAAGAAGCATCCAGA GC and reverse: GAACTTGGTGGCGTTGGAGAC TC; NQO1: forward, CAGTGGCATGCACCCAGG-GAA and reverse, GCATGCCCCTTTTAGCCTTGG CA; CYP450 1A5: forward, TCACCATCCCGCACAG CA and reverse, AAGTCATCACCTTCTCCGCATC;

CYP450 3A37: forward, CGAATCCCAGAAATCAGA and reverse, AGCCAGGTAACCAAGTGT; Beclin-1: forward, CGACTGGAGCAGGAAGAAG and reverse: TCTGAGCATAACGCATCTGG; ATG-5: forward, GGCACCGACCGATTTAGT and reverse: GCTGAT GGGTTTGCTTTT; LC3-I: forward, TTACACCCA-TATCAGATTCTTG and reverse: ATTCCAACCTGT CCCTCA; LC3-II: forward, CTTCTTCCTCCTGGTG AACG and reverse, GCACTCCGAAAGTCTCCTGA; Caspase9: forward, GAAGGGAGCAAGCACGAC and reverse, GGTTGGACTGGGATGGAC; Caspase3: forward, AAGGCTCCTGGTTTATTC and reverse, CTG CCACTCTGCGATTTA; β-actin: forward, TGAAGC CCAGAGCAAAAGAG and reverse, TGCTCCTCAG GGCTACTCTC. The gene expression was determined by RT-qPCR, the reaction condition was: pre-denaturation 94°C for 5 min, 35 cycles of denaturation 94°C for 30 s, annealing 60°C for 30 s, and extension 72°C for 30 s. β-actin was used as an internal reference, the relative expression of each gene was calculated by the $2^{-\Delta\Delta Ct}$ method.

Western Blot

The total cell protein was extracted by PMSF: RIPA (1: 100) buffer, protein concentration was measured by BCA kit. The equal amount of protein from each sample was separated by SDS-PAGE electrophoresis, transferred to PVDF membranes, and blocked with 5 % skim milk for 1 h. The PVDF membranes were washed with TBST, and incubated with primary antibodies (rabbit anti-HO-1, NQO1, Keap1, Nrf2) for overnight at 4°C. The PVDF membranes were washed with TBST, and incubated with goat anti-rabbit HRP-IgG secondary antibodies for 1 h at room temperature. The protein signals were detected by ECL kit, the relative expression levels of the target proteins in each group were analyzed by Image J software, and β-actin was used as an internal reference.

Fluorescence Microscopy for Cell Autophagy by Cyto-ID

The cells were washed with 1 × assay buffer, incubated with staining solution (1 mL 1 × assay buffer containing 5% FBS + 2 μL Cyto-ID green + 1 μL Hoechst 33342) at 37°C for 30 min, fixed with 4% paraformaldehyde for 20 min, and washed with 1 × assay buffer. The cell autophagy was observed by the inverted fluorescence microscopy.

Flow Cytometry for Cell Apoptosis by Annexin V-FITC

The cells were digested with trypsin and centrifuged, adjusted to a density of 5×10^5 cells/mL with binding buffer, incubated with Annexin V-FITC in the dark for 10 min and washed with Binding buffer. PI was added into the cells, cell apoptosis was determined by flow cytometry assay.

Statistical Analysis

The experimental data were expressed as means ± SD, and analyzed by one-way ANOVA and student's t-test using SPSS 20.0 software (SPSS, Inc., Chicago, IL). P value < 0.05 indicated to be a statistical significant difference.

RESULTS AND DISCUSSION

Taraxasterol Alleviates Oxidative Stress by Activating Nrf2/Keap1 Signaling Pathway in AFB1-Induced Chicken Hepatocytes

The isolated cells from chicken embryos were identified as primary hepatocytes by glycogen PAS staining. The toxic dose of AFB1-induced hepatocytes was 0.05 μg/mL by the analysis of the cell viability and ALT and AST activities. Zero to 20 μg/mL taraxasterol had no effect on the cell viability by MTT analysis, thus 5, 10, and 20 μg/mL taraxasterol was used in the following experiments, respectively.

GSH, SOD, ROS, and MDA are the most important indicators related to oxidative stress. To evaluate the anti-oxidative capacity of taraxasterol on AFB1-induced chicken hepatocytes, we analyzed the levels of SOD, GSH, MDA and ROS. The results showed that SOD and GSH activity in AFB1 group was decreased significantly ($P < 0.01$), and the MDA and ROS content in AFB1 group was increased significantly ($P < 0.01$) compared with those in normal group (Figure 1A). However, the activity of SOD and GSH in taraxasterol groups was increased significantly ($P < 0.05$ or $P < 0.01$) compared with those in AFB1 group; while the production of MDA and ROS was decreased significantly in taraxasterol groups compared with those in AFB1 group ($P < 0.01$) (Figure 1A). Our previous studies have shown that taraxasterol protected against ethanol-induced and Con A-induced liver injuries by increasing hepatic GSH and SOD levels, and decreasing hepatic MDA and ROS production in mice (Xu et al., 2018; Sang et al., 2019), the present study was in line with our previous work.

Nrf-2/Keap1 pathway is an adaptive cellular response conferring protection against oxidative stress (Vriend and Reiter, 2015). AFB1 activates ROS production in broiler liver, causing oxidative stress and participating in the Nrf2 signaling pathway (Wang et al., 2018). To further explore the anti-oxidative mechanism of taraxasterol on AFB1-induced chicken hepatocytes, we studied the mRNA and protein expression levels of key factors HO-1, NQO1, Nrf2 and Keap1 in Nrf2/Keap1 pathway. Compared with normal group, the mRNA and protein expression levels of HO-1, NQO1, and Nrf2 in AFB1 group were significantly decreased ($P < 0.01$), and the mRNA and protein levels of Keap1 in AFB1 group were significantly

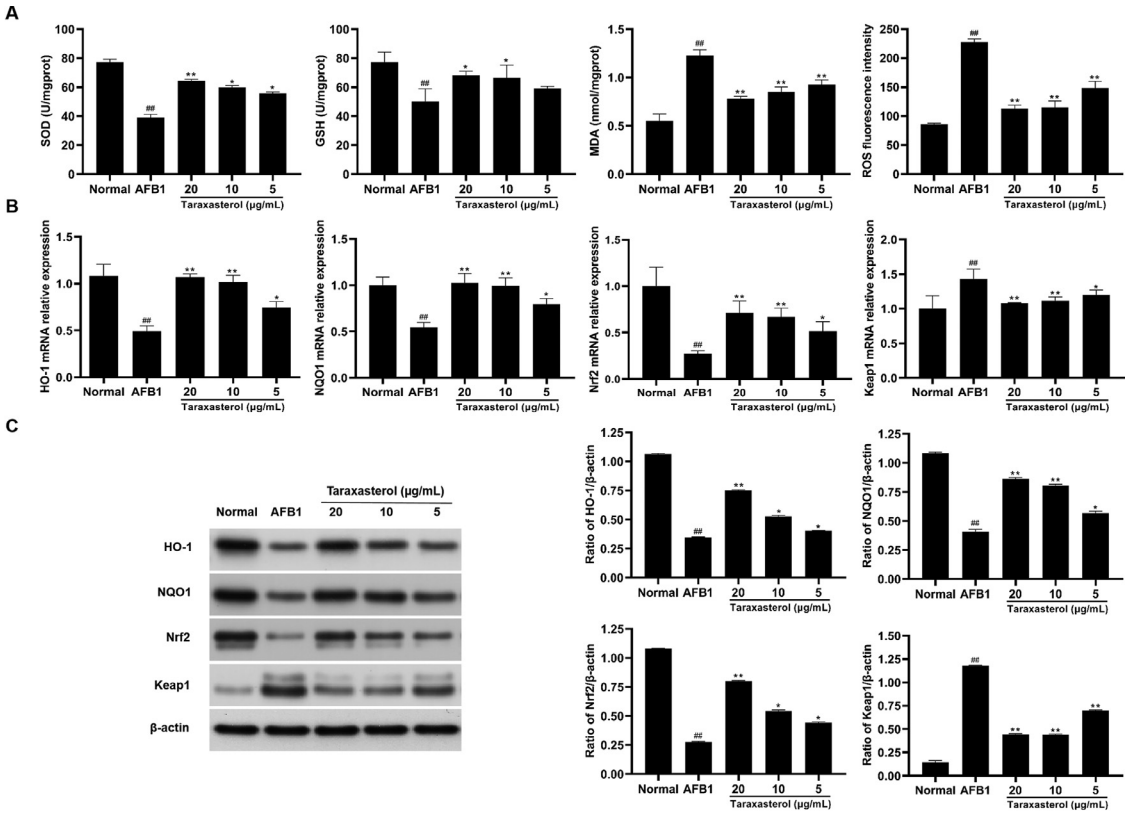

Figure 1. Effects of taraxasterol on oxidative stress by regulating Nrf2/Keap1 signaling pathway in AFB1-induced chicken hepatocytes. The levels of SOD, GSH, MDA and ROS (A) and the mRNA (B) and protein (C) expression of HO-1, NQO1, Nrf2 and Keap1 were determined. Data represent the means ± SD for n = 5. $^{\#\#}P < 0.01$ vs. normal group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. AFB1 group.

increased ($P < 0.01$) (Figure 1B and C). Compared with AFB1 group, the mRNA and protein expression of HO-1, NQO1 and Nrf2 in taraxasterol groups was significantly increased ($P < 0.05$ or $P < 0.01$), and the mRNA and protein expression of Keap1 in taraxasterol groups was significantly decreased ($P < 0.05$ or $P < 0.01$) (Figure 1B and C). The Western blot results were consist with the RT-qPCR results. These results indicated that taraxasterol activated the Nrf2/Keap1 anti-oxidation pathway in AFB1-induced chicken hepatocytes.

Taraxasterol Regulates the Cell Autophagy and Apoptosis in AFB1-Induced Chicken Primary Hepatocytes

Oxidative stress is closely associated with autophagy, AFB1 promotes autophagy associated with oxidative stress-related signaling pathway (Huang et al., 2019). In this experiment, fluorescence microscopy results showed that AFB1 induced high autophagy fluorescence intensity, while taraxasterol decreased autophagy fluorescence intensity (Figure 2A). The RT-qPCR results further showed that AFB1 significantly induced the expression of autophagy-related genes Beclin-1, ATG-5, and LC3-I/LC3-II ($P < 0.01$), while taraxasterol significantly inhibited the expression of these genes induced by AFB1 ($P < 0.01$) (Figure 2B), indicating that taraxasterol protected against AFB1-induced hepatocyte autophagy by inhibiting the expression of autophagy-related genes.

ROS produced by oxidative stress may also activate the apoptosis signaling pathway, and trigger hepatocyte apoptosis. Caspase cascade system plays an important role in the induction, transduction and amplification of intracellular apoptosis signals (Kesavardhana et al., 2020). Flow cytometry results showed that the apoptosis rate of hepatocytes in AFB1 group was significantly increased compared with normal group ($P < 0.01$), while the apoptosis rates in taraxasterol groups were significantly decreased compared with AFB1 group ($P < 0.01$) (Figure 2C). Furthermore, the mRNA expression of Caspase3 and Caspase9 in AFB1 group was significantly increased compared with normal group ($P < 0.01$); while the mRNA expression of Caspase3 and Caspase9 in taraxasterol groups was significantly decreased compared with AFB1 group ($P < 0.05$ or $P < 0.01$) (Figure 2D), indicating that taraxasterol played a protective role against AFB1-induced chicken hepatocyte apoptosis by inhibiting the expression of apoptotic genes.

In summary, our results indicated that taraxasterol alleviated AFB1-induced oxidative stress in chicken primary hepatocytes by increasing SOD and GSH activity and decreasing ROS and MDA production. The alleviative mechanism was associated with activating the Nrf2/Keap1 signaling pathway, inhibiting hepatocyte apoptosis and autophagy via regulating the apoptosis and autophagy-related genes.

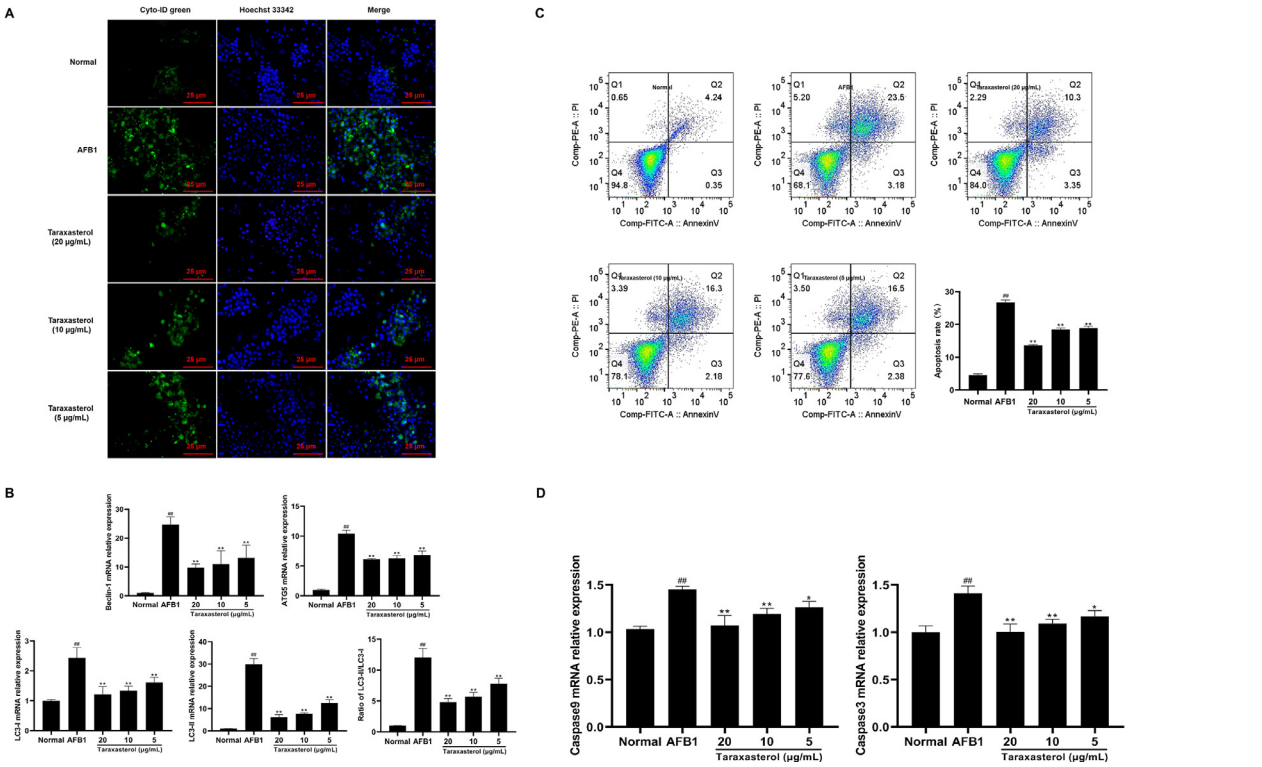

Figure 2. Effects of taraxasterol on autophagy and apoptosis in AFB1-induced chicken hepatocytes. The cell autophagy (A, 200 ×, green fluorescence: autophagy cells), the mRNA expression of autophagy-related genes Beclin-1, ATG-5, LC3-I, LC3-II, LC3-II/LC3-I (B), the cell apoptosis and apoptosis rate (C), and the mRNA expression of apoptosis-related genes Caspase3 and Caspase9 (D) were determined. Data represent the means ± SD for n = 5. $^{\#\#}P < 0.01$ vs. normal group; $^{*}P < 0.05$, $^{**}P$.

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DISCLOSURES

The authors declare that they have no conflicts of interest.

REFERENCES

Huang, W. Y., Z. Cao, J. Zhang, Q. Ji, and Y. F. Li. 2019. Aflatoxin B1 promotes autophagy associated with oxidative stress-related PI3K/AKT/mTOR signaling pathway in mice testis. Environ. Pollut. 255:113317.

Kesavardhana, S., R. K. S. Malireddi, and T. D. Kanneganti. 2020. Caspases in cell death, inflammation, and gasdermin-induced pyroptosis. Annu. Rev. Immuno. 14:567–595.

Li, S. H., R. M. Liu, G. Q. Wei, G. F. Guo, H. X. Yu, Y. X. Zhang, M. Ishfaq, S. A. Fazilani, and X. Y. Zhang. 2021. Curcumin protects against Aflatoxin B1-induced liver injury in broilers via the modulation of long non-coding RNA expression. Ecotoxicol. Environ. Saf. 208:111725.

Peles, F., P. Sipos, Z. Győri, W. P. Pfliegler, F. Giacometti, A. Serraino, G. Pagliuca, T. Gazzotti, and I. Pócsi. 2019. Adverse effects, transformation and channeling of aflatoxins into food raw materials in livestock. Front. Microbio. 10:2861.

Rushing, B. R., and M. I. Selim. 2019. Aflatoxin B1: a review on metabolism, toxicity, occurrence in food, occupational exposure, and detoxification methods. Food Chem. Toxicol. 124:81–100.

Sang, R., Y. F. Yu, B. J. Ge, L. Xu, Z. Wang, and X. M. Zhang. 2019. Taraxasterol from *Taraxacum* prevents concanavalin A-induced acute hepatic injury in mice via modulating TLRs/NF-κB and Bax/Bc1-2 signalling pathways. Artif. Cells Nanomed. Biotechnol. 47:3929–3937.

Vriend, J., and R. J. Reiter. 2015. The Keap1-Nrf2-antioxidant response element pathway: a review of its regulation by melatonin and the proteasome. Mol. Cell. Endocrinol. 401:213–220.

Wang, H., I. Muhammad, W. Li, X. Q. Sun, P. Cheng, and X. Y. Zhang. 2018. Sensitivity of Arbor Acres broilers and chemoprevention of aflatoxin B1-induced liver injury by curcumin, a natural potent inducer of phase-II enzymes and Nrf2. Environ. Toxicol. Pharmacol. 59:94–104.

Xu, L., Y. F. Yu, R. Sang, J. X. Li, B. J. Ge, and X. M. Zhang. 2018. Protective effects of taraxasterol against ethanol-induced liver injury by regulating CYP2E1/Nrf2/HO-1 and NF-κB signaling pathways in mice. Oxid. Med. Cell. Longev. 2018:8284107.

Zhao, L., L. Zhang, Z. J. Xu, X. D. Liu, L. Y. Chen, J. F. Dai, N. A. Karrow, and L. H. Sun. 2021. Occurrence of Aflatoxin B1, deoxynivalenol and zearalenone in feeds in China during 2018-2020. J. Anim. Sci. Biotechnol. 12:74.