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Molybdenum and cadmium co-induce apoptosis and ferroptosis through inhibiting Nrf2 signaling pathway in duck (*Anas platyrhyncha*) testes

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ABSTRACT Cadmium (**Cd**) and high molybdenum (**Mo**) are injurious to the body. Previous research has substantiated that Cd and Mo exposure caused testicular injury of ducks, but concrete mechanism is not fully clarified. To further survey the toxicity of co-exposure to Cd and Mo in testis, 40 healthy 8-day-old Shaoxing ducks (*Anas platyrhyncha*) were stochasticly distributed to 4 groups and raised with basic diet embracing Cd (4 mg/kg Cd) or Mo (100 mg/kg Mo) or both. At the 16th wk, testis tissues were gathered. The characteristic ultrastructural changes related to apoptosis and ferroptosis were observed in Mo or Cd or both groups. Besides, Mo or Cd or both repressed nuclear factor erythroid 2-related factor 2 (**Nrf2**) pathway via decreasing Nrf2, Heme oxygenase-1 (**HO-1**), NAD(P)H quinone oxidoreductase 1 (**NQO1**), Glutamate-cysteine ligase catalytic subunit (**GCLC**) and Glutamate-cysteine ligase modifier subunit (**GCLM**) mRNA expression of and Nrf2 protein expression, then stimulated apoptosis by elevating Bcl-2 antagonist/killer-1 (**Bak-1**), Bcl-2-associated X-protein (**Bax**), Cytochrome complex (**Cyt-C**), caspase-3 mRNA expression, cleaved-caspase-3 protein expression and apoptosis rate, as well as reducing B-cell lymphoma-2 (**Bcl-2**) mRNA expression and ratio of Bcl-2 to Bax, and triggered ferroptosis by upregulating Acyl-CoA Synthetase Long Chain Family Member 4 (**ACSL4**), transferrin receptor (**TFR1**) and Prostaglandin-Endoperoxide Synthase 2 (**PTGS2**) expression levels, and downregulating ferritin heavy chain 1 (**FTH1**), ferritin light chain 1 (**FTL1**), ferroportin 1 (**FPN1**), solute carrier family 7 member 11 (**SCL7A11**) and glutathione peroxidase 4 (**GPX4**) expression levels. The most obvious changes of these indexes were observed in co-treated group. Altogether, the results announced that Mo or Cd or both evoked apoptosis and ferroptosis by inhibiting Nrf2 pathway in the testis of ducks, and co-exposure to Mo and Cd exacerbated these variations.

Key words: molybdenum, cadmium, apoptosis, ferroptosis, testis

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INTRODUCTION

Molybdenum (**Mo**) holds a substantial importance as a metal element with characteristics such as elevated melting point, exceptional strength, and effective corrosion resistance. Additionally, it is also a vital trace mineral for humans, animals and plants (Novotny and Peterson, 2018). However, an excess of Mo is detrimental to physiological health. Mo is extensively applied in industrial and agricultural production and animal feed, which increases its risk of entering the environment. Permissible level of Mo in potable water, as stated by the World Health Organization (**WHO**), was reported to not exceed 70 μg/L (Organization, 1996). Mo content of drinking water in some areas of China, Argentina and the United States reached 113 μg/L, 90 μg/L and 211 μg/L, respectively (Chen et al., 2020; Lawson-Wood et al., 2021; Arienzo et al., 2022). Some literatures have substantiated that high Mo can cause damage in kidneys, livers, spleens, and ovaries (Cao et al., 2016; Shi et al., 2017; Dai et al., 2018a; Guo et al., 2022). Besides, testicle is also target organ of Mo toxicity, and excessive Mo could cause its structural changes, decrease sperm quantity and quality, unbalance sex hormone levels, and weaken reproductive capacity (Asadi et al., 2017). Mao et al., (2022) reported that high Mo inhibited cancer cell proliferation by evoking ferroptosis and apoptosis.

Cadmium (**Cd**), a metal element with harmful properties, pervades environment widely (Lamraoui et al., 2022). Its pollution sources mainly include the

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development of mineral resources, industrial "3 wastes" and automobile exhaust and so on. Cd pollution has become a worldwide environmental problem. The incidence of severe pollution, the rate of exceeding established standards and average concentration of Cd in Chinese paddy soils reached 8.6, 33.2, and 0.23 mg/kg, respectively (Liu et al., 2016). A large amount of industrial wastewater containing high concentration of Cd is discharged into the water, which poses a considerable risk to animal health via the food chain (Gong et al., 2022). The increasing evidence attested that reproductive organ, especially testicle, is extremely sensitive to Cd toxicity (Shi and Fu, 2019; da Silva et al., 2021). Cd could lead to sperm count reduce and abnormal morphological structure in testicles (Venditti et al., 2021; Zhou et al., 2022). Zeng et al., (2021) showed that Cd impaired testicular function by promoting ferroptosis. Besides, some studies borne out that Cd could cause oxidative stress, apoptosis and ferroptosis in testicles (Zhang et al., 2012; Wang et al., 2023).

As a regulated cell death process, apoptosis is triggered through organism in response to external stimuli (Newton et al., 2024). Oxidative stress is a principal factor of triggering apoptosis. As a protective antioxidant transcription factor, the nuclear factor erythroid 2-related factor 2 (**Nrf2**) has a crucial action in antioxidant defense response during oxidative stress (Yamamoto et al., 2018). Study demonstrated that Nrf2 signaling pathway had a strong anti-apoptotic effect (Ma et al., 2022). It could inhibit B-cell lymphoma-2 (**Bcl-2**) expression and promote Bcl-2-associated X-protein (**Bax**) expression (Deng et al., 2021; Hu et al., 2021). Previous study confirmed that Cd exposure evoked apoptosis through inhibiting Nrf2 signaling pathway in the testicle of rats (Kassab et al., 2020). Additionally, Mo exposure was found to cause hepatotoxicity in ducks by disrupting Nrf2 signaling pathway(Wang et al., 2022b).

Ferroptosis constitutes a novel variant of programmed cellular demise, characterized by its reliance on iron (Dixon et al., 2012). Its primary cause is the excessive build-up of lipid peroxidation dependent on iron. Characteristic morphological attributes of ferroptosis encompass contraction of mitochondrial membrane, augmented density of the same, and diminishment or vanishing of mitochondrial cristae. It is regulated by many genes, such as glutathione peroxidase 4 (**GPX4**), solute carrier family 7 member 11 (**SCL7A11**), ferritin light chain 1 (**FTL1**), ferritin heavy chain 1 (**FTH1**), transferrin receptor (**TFR1**) and ferroportin 1 (**FPN1**). Besides, Nrf2 signaling pathway is of utmost importance in the regulation of ferroptosis (Dodson et al., 2019). Study has found that both SCL7A11 and GPX4 can be upregulated by activating Nrf2 signaling pathway, which can directly decompose lipid peroxidation products (Dong et al., 2023). Many proteins participate in iron storage and transport, such as FTH1, FTL1, TFR1 and FPN1, are also regulated by Nrf2 (Wang et al., 2022c). Therefore, activating Nrf2 pathway may reduce excessive iron accumulation and prevent ferroptosis. Cd caused ferroptosis in the testicle of mice by restraining Nrf2 signaling pathway (Xiong et al., 2022).

As modern industry continues development, the widespread emission of heavy metals has emerged as a global environmental concern. The common occurrence of heavy metals joint entering the environment has elicited serious impacts on both the ecological environment and human health. Therefore, the research of combined heavy metals' toxicity has captured the interest of many researchers. Mo and Cd are often associated with other minerals such as tungsten ores. During the mining process of tungsten ores, incomplete processing can result in the environment experiencing the simultaneous release of Mo and Cd, which eventually leads to combined pollution. Compared with other mammals, a large portion of waterfowl primarily feed in paddy fields and rivers, and it is more prone to the external environment and more susceptible to diseases. Shaoxing duck (*Anas platyrhyncha*) is the main breed in Southern China, which is also the main breed in the areas polluted by Mo and Cd. The duck has many advantages such as high laying rate, long egg production peak duration, high feed utilization rate, and strong life force. It ranks as one of the most favored breeds within China. Consequently, this species was used in the experiment. Recent investigations have demonstrated that the toxicity of Cd and Mo are far higher than the toxicity of their single pollution (Zhang et al., 2021; Chu et al., 2023). Previous results have certificated that combined exposure to Cd and Mo leads to testicular damage in ducks (Pu et al., 2023), but toxic mechanism has not been well assessed. Therefore, subchronic poisoning models of ducks that were exposed to either Mo, Cd, or both were established in this research, and the united toxic impacts of Cd and Mo on testes were discussed from the perspectives of Nrf2 signaling pathway, apoptosis and ferroptosis. The intent of this study was to heighten awareness regarding ecological understanding and the perils heavy metals pose to birds, offering a theoretical basis for reproductive toxicological research on co-exposure to Cd and Mo.

MATERIALS AND METHODS

Animals and Treatment

All animal contracts and manipulations were confirmed by Institutional Animal Care and Use Committee of Jiangxi Agricultural University (NO. JXAULL-2020-23). In this study, a total of 40 Shaoxing ducks (*Anas platyrhyncha*) aged 1-d were purchased from a local hatchery (Jiangxi, China) and were acclimated for 1 wk. Four groups were established from the population of 8-day-old ducks and distributed randomly. Each group had a different dietary intake, with varying amounts of Cd or Mo or both added to their basal feed per kilogram: a control group (receiving no Mo or Cd), a group given 100 mg of Mo (labelled as the Mo group), a group given 4 mg of Cd (the Cd group), and a group receiving both (the Mo + Cd group). In this research, $3CdSO_4{\cdot}8H_2O$ (Aladdin, China) and $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (Aladdin, China) were picked as the sources of Cd and Mo, respectively. The dosages of Mo and Cd were chosen according

to previous researches (Cui et al., 2021; Guo et al., 2022). The Table S1 enumerated the fundamental constituents of the diet. The ducks were housed in separate cages and allowed to eat and drink freely.

Sample Collection

After the culmination of the 16 wk of rearing, the ducks were slaughtered. Posteuthanasia, the testes from each individual duck were carefully extracted. Subsequently, both testes were rinsed 2 to 3 times with 0.9% NaCl. One testis was kept at -80 °C for RNA isolation and total protein extraction. The other testis was fixed in 2.5% glutaraldehyde and 4% formalin for ultrastructural histopathological, and immune-histochemical studies, respectively.

Ultrastructural Observation

Referring to former method (Liu et al., 2014), after fixed with 2.5% glutaraldehyde, testis tissues were stained with methanolic uranyl acetate and lead citrate. At last, the changes of testicular ultrastructure were assessed with a transmission electron microscope (**TEM**) Zeiss 900 (Zeiss, Germany).

TUNEL Assay

The method of TUNEL detection referred to Guo et al. (2022) study. DAPI was employed to label the nucleus, which consequently appeared blue. Apoptotic cells exhibited positive results that showed a green hue. Subsequently, the average proportion of apoptotic cells was computed for every group.

Ferrous Iron Assay in Testes

The ferrous iron level was assessed in testes according to ferrous iron colorimetric test kit (Elabscience, Wuhan). Testicular tissues (0.1g) were homogenized with 10 mL of iron assay buffer and then spun at 10,000 rcf for 10 min to collect the supernatant. The 300 μL sample and standard were added to 1.5 mL EP tubes respectively, followed by the addition of 150 μL of color development solution to each EP tube. The contents of the EP tubes were thoroughly mixed and maintained at 37°C for 30 min. Afterward, the tubes were spun at 12,000 rcf for 10 min. Utilizing a microplate reader, the absorbance of every individual well was recorded at a wavelength of 593 nm.

Real-Time Quantitative PCR analysis

According to previous method (Dai et al., 2019), total RNA was extracted, then reverse transcribed to cDNA using TaKaRa (Japan) kit. 2 × SYBR Green qPCR Master Mix (None ROX, Servicebio, Wuhan) was used in real-time quantitative PCR (**RT-qPCR**) on Bio-Rad's CFX Connect System. Glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) was applied as endogenous control and relative mRNA levels were computed by applying $2^{-\Delta\Delta CT}$ method. The Table S2 presented primer sequences.

Western Blotting Analysis

A previously established method was employed to extract total protein from testis tissues (Liu et al., 2022a). Testicular tissues were homogenized in a homogenizer after the addition of protein lysate. Following lysis at 4°C for 30 min, the mixture was centrifuged and the supernatant was aspirated as a total protein sample. Additionally, nuclear proteins were extracted using a Nucleoprotein Extraction Kit (Solarbio Biotechnology, Beijing, China). The protein concentration was then determined using the BCA protein assay kit (Solarbio Biotechnology, Beijing, China). The volume of the sample was determined based on the total amount of protein. Finally, the protein samples underwent electrophoresis and western blot analysis. The primary antibodies were GPX4 (1:1,000), FTH1 (1:2000), SCL7A11 (1:1,000), Acyl-CoA Synthetase Long Chain Family Member 4 (**ACSL4**) (1:1,000), TFR1 (1:1,000), FPN1 (1:1,000), Prostaglandin-Endoperoxide Synthase 2 (**PTGS2**) (1:1,000), cleaved-caspase-3 (1:500), Nrf2 (1:500) and GAPDH (1:5,000). GPX4, FTH1, SCL7A11, ACSL4, TFR1, and FPN1 came from Abmart (China). PTGS2, cleaved-caspase-3 and Nrf2 were sourced from Wanleibio (China). GAPDH was obtained from Proteintech (China). The GAPDH band was utilized for standardized quantitative analysis.

Statistical Analysis

The experimental results were presented as mean ± standard deviation. Statistical analysis was executed by using one-way analysis of variance (**ANOVA**) method of SPSS 25.0 software. To draw diagrams, GraphPad Prism 7.0 (GraphPad Software, San Diego, CA) and Image J software (National Institutes of Health, DC) were utilized. And Origin 2022 software (OriginLab, Northampton, Massachusetts, USA) was used to do correlation analysis. Statistical significance was determined as $P < 0.05$. *, ** and *** indicated significant differences at $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared with the control group. $^{\#}$, $^{\#\#}$ and $^{\#\#\#}$ indicated significant differences at $P < 0.05$, $P < 0.01$ and $P < 0.001$ between corresponding groups.

RESULTS

Cd or Mo or Both Injured the Ultrastructure of Testes

Testicular ultrastructure was showed in Figure 1. The control group did not exhibit any significant pathological changes, with intact nuclei and organelles. In the single treatment group, mitochondrial cristae rupture and ridge reduction occurred. The most severe pathological damage was revealed in the Mo + Cd group, which

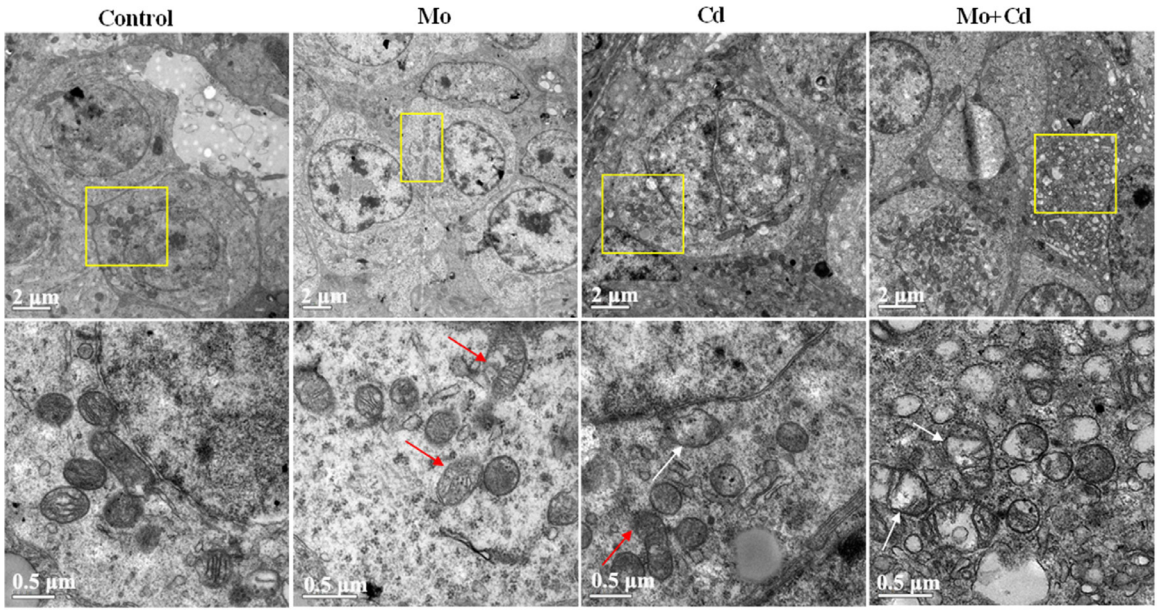

Figure 1. Mo or Cd or both caused ultrastructural damage of testis in ducks. Red arrow: rupture of mitochondrial membrane; white arrow: disappearance of mitochondrial cristae, scale bar = 2 μm and 0.5 μm.

showed mitochondrial vacuolization, rupture of outer membrane, and an apparent increase in the number of cristae reduction compared with the single groups.

Cd or Mo or Both Inhibited Nrf2 Pathway in Testes

As presented in Figures 2A and 2B, the mRNA levels of Nrf2, NAD(P)H quinone oxidoreductase 1 (**NQO1**), Heme oxygenase-1 (**HO-1**), Glutamate-cysteine ligase modifier subunit (**GCLM**)and Glutamate-cysteine ligase catalytic subunit (**GCLC**) in Mo or Cd or both groups were reduced ($P < 0.05$) compared with the control group. The mRNA levels of aforementioned genes of the co-treated group showed a decrease ($P < 0.05$) compared with the single-treated groups, except for GCLC and GCLM mRNA levels in the Cd group. Total Nrf2 protein level and nuclear Nrf2 protein level in the Mo or Cd or both groups were reduced ($P < 0.05$) compared with the control group (Figure 2C and 2D). The co-treatment group exhibited the most significant alterations among these observed changes.

Mo or Cd or Both Triggered Apoptosis in Testes

Figures 3A and B illustrated the results of TUNEL staining and showcased the differences in the number of

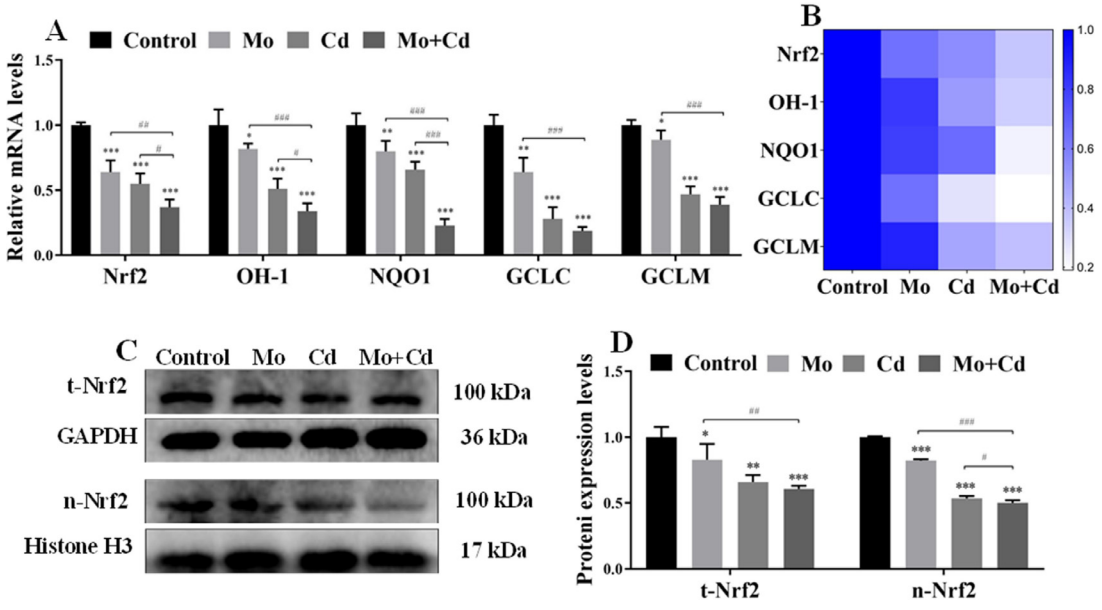

Figure 2. Mo or Cd or both inhibited Nrf2 pathway in the testis of ducks. (A) The mRNA expression levels of Nrf2 pathway-related genes; (B) Heatmap analysis of Nrf2 pathway-related genes mRNA expression levels; (C, D) The protein expression level of Nrf2. Note: *, ** and *** indicate significant differences at $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared with the control group. $^{\#}$, $^{\#\#}$ and $^{\#\#\#}$ indicate significant differences at $P < 0.05$, $P < 0.01$ and $P < 0.001$ between corresponding groups. Below is the same.

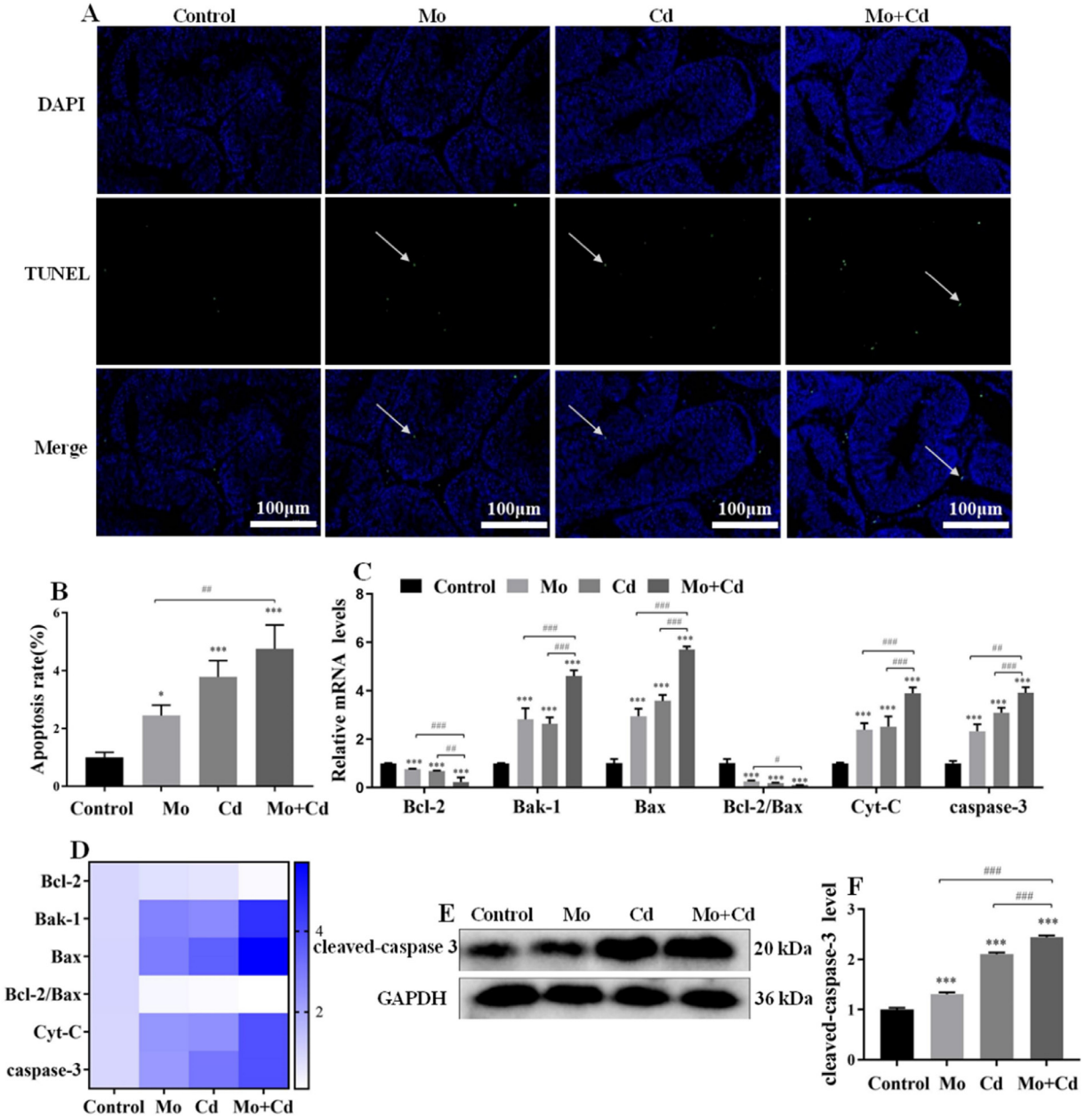

Figure 3. Mo or Cd or both induced apoptosis in the testis of ducks. (A) TUNEL staining (scale bar = 100 μm), white arrow: positive cells; (B) Apoptosis rate; (C) The mRNA expression levels of apoptosis-related genes; (D) Heatmap analysis of apoptosis-related genes mRNA expression levels; (E, F) The protein expression level of cleaved-caspase-3.

apoptosis cells between treated groups and control group. Compared with the control group, increased ($P < 0.05$) apoptosis cell numbers in the Mo or Cd or both treated groups were observed. Furthermore, a higher number of apoptosis cells were observed in the combined group.

As described in Figure 3C, in comparison with control group, there was a upregulation ($P < 0.05$) in Bax, Bcl-2 antagonist/killer-1 (**Bak-1**), caspase-3 and Cytochrome complex (**Cyt-C**) mRNA expression of Mo or Cd or both groups. Conversely, Bcl-2 mRNA expression and ratio of Bcl-2 to Bax exhibited downregulation ($P < 0.05$). The biggest alterations were presented in the co-treated group. In addition, the genes mRNA expression was visualized and analyzed using heatmap (Figure 3D). Compared to control group, cleaved-caspase-3 protein level was upregulated ($P < 0.05$) in the 3 treated groups (Figures 3E−3F). The alterations of these indicators in combined group were most evident.

Cd or Mo or Both Triggered Ferroptosis in Testes

As illustrated in Figure 4A, compared to control group, the treated groups showed an elevation ($P < 0.05$) in the concentration of ferrous iron in testes, and it was pronounced ($P < 0.001$) in united group. Figure 4B displayed mRNA transcript levels of genes associated with ferroptosis. Compared to control group, ACSL4, TFR1, and PTGS2 mRNA levels were elevated ($P < 0.05$) in the 3 treated groups, except for the PTGS2

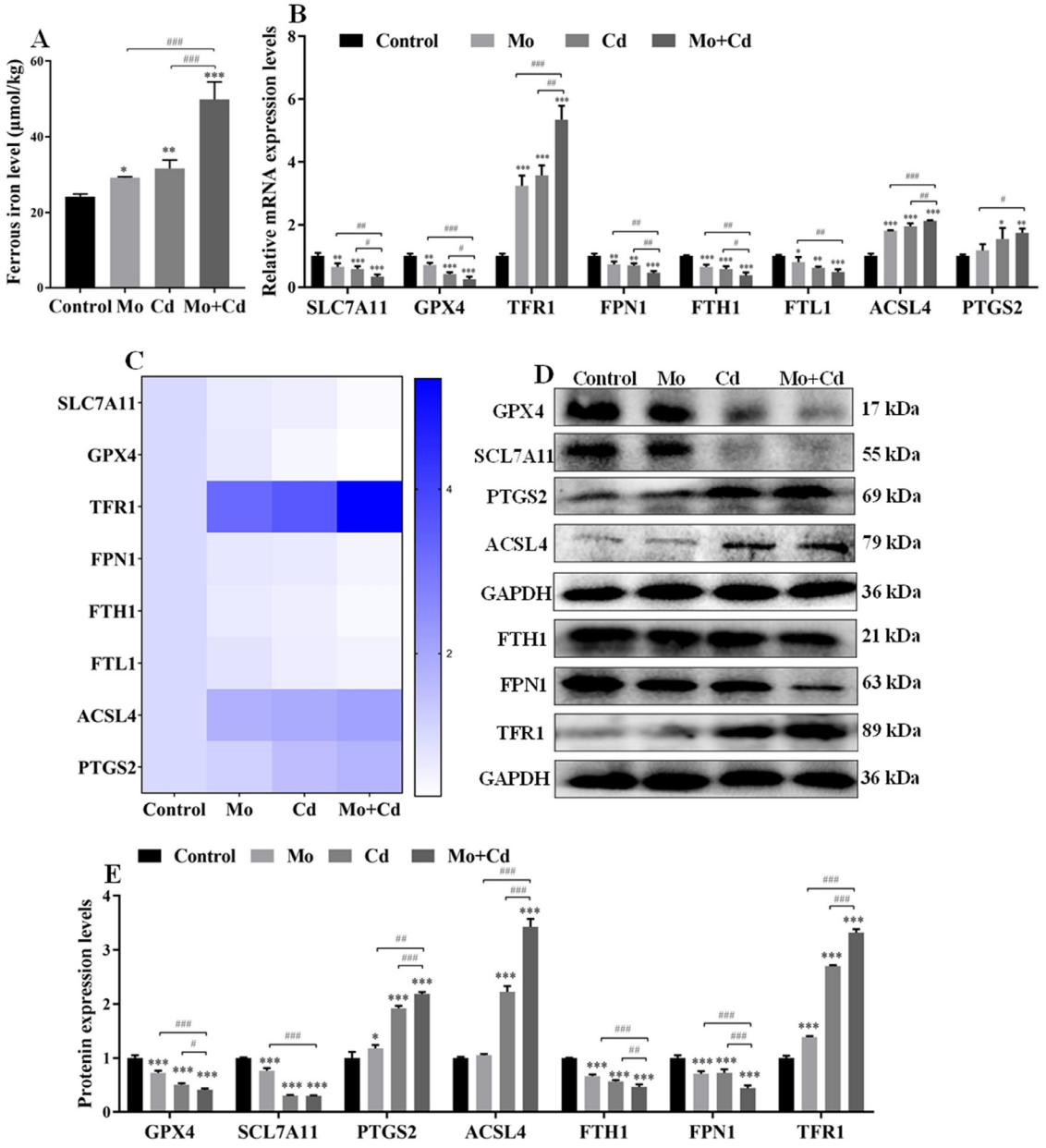

Figure 4. Mo or Cd or both induced ferroptosis in the testis of ducks. (A) The level of ferrous ion; (B) Ferroptosis-related genes mRNA expression levels; (C) Heatmap analysis of ferroptosis-related genes mRNA expression levels; (D, E) The expression levels of ferroptosis-related proteins.

mRNA level in Mo group. Except for the PTGS2 mRNA level in the Cd group, these genes mRNA levels were the highest in Mo + Cd group. In contrast, compared with control group, FTH1, FTL1, FPN1, SCL7A11 and GPX4 mRNA levels in Mo or Cd or both groups were decreased ($P < 0.05$), and reduced ($P < 0.05$) in Mo + Cd group compared with alone treated groups, except for FTL1 mRNA expression in the Cd group. In addition, Figure 4C depicted the heatmap analysis of the genes. In accordance with the Figures 4D−4E, western blotting analysis was performed to assay ferroptosis markers protein levels. Except for ACSL4 protein level in the Mo group, PTGS2, ACSL4 and TFR1 protein levels in all the treated groups were upregulated ($P < 0.05$) compared to control group, GPX4, FTH1, SCL7A11 and FPN1 protein levels were downregulated ($P < 0.05$). Furthermore, the most pronounced changes in these protein levels were presented in Mo + Cd group.

Correlation Analysis

The relationship between Nrf2 pathway, apoptosis, and ferroptosis was further confirmed through correlation analysis. As depicted in Figure 5, an obvious negative correlation was observed between mRNA transcription levels of Nrf2 pathway (Nrf2, NQO1, HO-1, GCLM, GCLC) and the mRNA transcription levels of apoptosis pertinent gene (Bcl-2) and ferroptosis related genes (GPX4, FTH1, FTL1, FPN1, SLC7A11), while it was markedly positively related to mRNA transcriptional levels of apoptosis pertinent genes (caspase-3, Bax, Cyt-C, Bak-1) and ferroptosis related genes (ACSL4, TFR1, PTGS2).

DISCUSSION

In recent years, combined pollution of heavy metals has emerged as a significant environmental problem

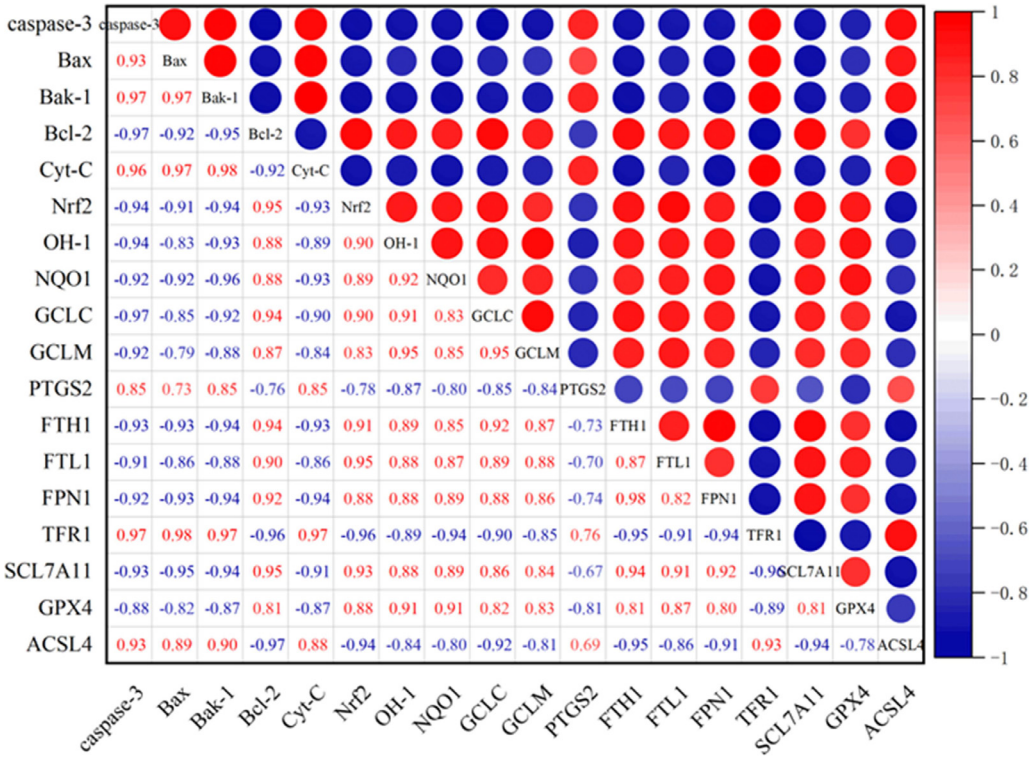

Figure 5. Correlation analysis between ferroptosis, apoptosis and Nrf2 pathway in the testis of ducks under Mo and Cd exposure. Note: The correlation coefficient of mRNA expression among the above 3 is shown as γ with values between −1 and 1, $\gamma > 0$ is a positive correlation, which is displayed in red, and $\gamma < 0$ is a negative correlation, which is displayed in blue.

and threatens public health. Among these, the concurrent contamination by Cd and Mo represents a quintessential instance. Dozens of studies have attested that Nrf2 signaling pathway, apoptosis and ferroptosis play important roles in the toxic mechanism of heavy metals (Qu et al., 2019; Irkin and Öztürk, 2022; Hu et al., 2023a). Testis is one of the important organs damaged by high Mo and Cd. Ducks often live and forage in the water, and are more susceptible to the external environment. Therefore, the toxic mechanism of Cd and Mo on duck testis was discussed from the perspectives of Nrf2 signaling pathway, ferroptosis and apoptosis. In the study, ultrastructural changes presented specific morphological characteristics related to apoptosis and ferroptosis in Cd or Mo or both exposure groups in duck testes, including mitochondrial cristae disappearance and even vacuolation, mitochondrial membrane rupture, and uniform round-shaped mitochondria, which indicates that apoptosis and ferroptosis may be key contributive testicular toxic mechanisms caused by Cd or Mo or both in ducks. Hence, their molecular mechanisms were further studied.

Nrf2 is an important nuclear transcription factor related to antioxidant responses. Under conditions of oxidative stress, the activation of Nrf2 prompts its translocation into the nucleus, initiating the transcriptional regulation of downstream antioxidant related factors such as HO-1, GCLC, NQO1, and GCLM, thereby renewing the redox balance of cells. HO-1 is a protective heme metabolism rate-limiting enzyme with various biological functions, including antioxidation, suppression of cell inflammation and apoptosis. NQO1 is pivotal in detoxification and metabolism, functioning to eradicate various oxidants. Maintaining cellular GSH homeostasis is reliant on GCL, which comprises GCLC and GCLM subunits. GCLC regulates GSH binding within cells, thereby activating the cellular detoxification mechanism. The efficacy of GCLC is doubled due to the direct interaction between GCLM and GCLC factors. Heavy metals have complex impacts on Nrf2 expression level, with effects varying by exposure duration and concentration (Buha et al., 2021). Short-term or low-dose exposure may transiently activate Nrf2 signaling pathway as a cellular defense (Cheng et al., 2022). However, prolonged or high-dose exposure can suppress Nrf2 signaling pathway, thereby compromising antioxidant defenses and increasing oxidative stress and cellular damage (Hu et al., 2023b). Study demonstrated that Mo or Cd or both inhibited Nrf2, HO-1, GCLC, GCLM, and NQO1 mRNA expression levels in duck livers (Wang et al., 2022b). The outcomes of this study were in line with the aforementioned results, suggesting there is a correlation between the toxicity of Cd or Mo or both and inhibition of Nrf2 signaling pathway in duck testes.

Apoptosis is an orderly cell death controlled through many genes and proteins (Bertheloot et al., 2021). Key proteins involved in the apoptosis by mitochondrial pathway include Bax and Bak-1. They promote the release of Cyt-C, which is a pivotal event that triggers the apoptotic cascade. Once Cyt-C enters the cytosol, it leads to the activation of caspase-3 through a series of reactions. Activated caspase-3, also called cleaved-caspase-3, is a critical executioner enzyme in apoptosis, which is responsible for the cleavage of various cellular

substrates, finally leading to apoptosis. On the other hand, Bcl-2 is an anti-apoptotic protein that helps maintain mitochondrial integrity and prevents the release of Cyt-C. It exerts its effect by inhibiting the activities of pro-apoptotic proteins like Bax and Bak-1. Thus, the ratio of Bcl-2 to Bax is often considered a determinant of cell survival or death. The data from this experiment revealed that treatment with Cd or Mo or both could increase Bak-1, Bax, caspase-3, and Cyt-C mRNA expression levels, cleaved caspase-3 protein expression level and apoptosis rate, and decrease Bcl-2 mRNA expression level and ratio of Bcl-2 to Bax. Previous studies have already disclosed that apoptosis activated by Mo or Cd or both is accompanied by an elevation in cleaved caspase-3 protein level and a decrease in ratio of Bcl-2 to Bax (Guo et al., 2022), which is consistent with the results of this experiment. Thus, these findings implied that both Cd and Mo independently could lead to mitochondrial pathway apoptosis in duck testes, and their combined exposure intensified these effects.

Ferroptosis is a cell death mechanism that depends on iron and lipid peroxidation. During ferroptosis, the interplay of some iron related factors contributes to the initiation and progression of this form of cell death(Jiang et al., 2021). FTH1 and FTL1 are involved in iron storage, whereas FPN1 and TFR1 regulate iron uptake and export. The balance of iron within the cell is critical, given that iron plays a role in generating reactive lipid peroxides, central to the induction of ferroptosis. GPX4, an antioxidant enzyme, protects against lipid peroxidation. SLC7A11 promotes glutathione synthesis to alleviate oxidative stress. ACSL4 and PTGS2 promote lipid peroxidation and exacerbate ferroptosis. An escalating body of research suggested that heavy metals induced ferroptosis (He et al., 2022; Ye et al., 2023). The data from this study suggested that Cd or Mo or both elevated ferrous ion content and expression levels of ACSL4, TFR1, and PTGS2 in testicular tissues, and reduced the expression levels of FTH1, FTL1, FPN1, SLC7A11, and GPX4. These findings are consistent with previous studies. In summary, Cd or Mo or both induced ferroptosis in duck testes, and their co-exposure intensified the impacts.

Numerous investigations have demonstrated a close association between the occurrence of apoptosis and ferroptosis with the Nrf2 signaling pathway (Dodson et al., 2019; Lian et al., 2023). According to some researches, the inhibition of Nrf2 signaling pathway could reduce the of Bcl-2 expression level and upregulate caspase family members' activities, thereby promoting the process of apoptosis (Dai et al., 2018b; Li et al., 2019; Cui et al., 2020). Many scholars found that Mo or Cd could induce apoptosis, which is highly relevant to restraining Nrf2 pathway (Koto et al., 2011; Guan et al., 2022). Additionally, study showed that Nrf2 assumed a vital role in modulating ferroptosis by regulation of some iron related factors' expression (Dodson et al., 2019). Activated Nrf2 promoted expression levels of FTH1, FTL1, GPX4, and SLC7A11, thus balancing iron level, reducing oxidative stress, preventing lipid damage, ultimately inhibiting ferroptosis (Wang et al., 2022a). Nrf2 also controlled iron transport through TFR1 and FPN1, and mitigated lipid peroxidation by modulating expression levels of ACSL4 and PTGS2 (Zhao et al., 2022). Growing evidence suggested that heavy metals might influence ferroptosis through Nrf2 signaling pathway (Liu et al., 2022b; Ouyang et al., 2023; Lan et al., 2024). In the present study, the correlation analysis of Figure 5 substantiated that Mo or Cd or both triggered ferroptosis and apoptosis via inhibiting Nrf2 signaling pathway in duck testes.

CONCLUSIONS

In summary, this study revealed that Cd- and Mo -induced apoptosis and ferroptosis were related to the inhibition of Nrf2 signaling pathway in the testes of ducks. Co-exposure to Mo and Cd exacerbated these changes.

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DISCLOSURES

The authors declare that they have no competing interests.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2024.103653.

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