

Toxicity mechanisms, exposure, toxicokinetic and risk assessment aspects of metals, toxic for animals and humans, volume III

Edited by

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Published in

Frontiers in Pharmacology



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ISSN 1664-8714
ISBN 978-2-8325-6926-9
DOI 10.3389/978-2-8325-6926-9

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Toxicity mechanisms, exposure, toxicokinetic and risk assessment aspects of metals, toxic for animals and humans, volume III

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Citation

Machado, M. M., El-Demerdash, F. M., Boye, A., Zhu, Y., Sun, X., Yang, X., eds. (2025). *Toxicity mechanisms, exposure, toxicokinetic and risk assessment aspects of metals, toxic for animals and humans, volume III*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-6926-9

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EDITED AND REVIEWED BY
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RECEIVED 21 August 2025
ACCEPTED 25 August 2025
PUBLISHED 16 September 2025

CITATION
Boye A, Zhu Y, Li X, Machado MM,
El Demerdash FM and Yang X (2025) Editorial:
Toxicity mechanisms, exposure, toxicokinetic
and risk assessment aspects of metals, toxic for
animals and humans, volume III.
Front. Pharmacol. 16:1690145.
doi: 10.3389/fphar.2025.1690145

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Editorial: Toxicity mechanisms, exposure, toxicokinetic and risk assessment aspects of metals, toxic for animals and humans, volume III

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KEYWORDS

exposure, metals, toxicity mechanisms, toxicokinetic, risk assessment

Editorial on the Research Topic

Toxicity mechanisms, exposure, toxicokinetic and risk assessment aspects of metals, toxic for animals and humans, volume III

Living organisms including humans and animals are unavoidably exposed to the chemical space (xenobiotics, e.g., chemicals in the air, water and the soil). Some xenobiotics such as food and drugs which are consciously consumed by humans and animals are useful in that they promote growth and development to impact health and wellbeing. However, other xenobiotics including but not limited to heavy metals (toxics) upon exposure to humans and animals may cause various degrees of toxicity affecting quality of human life and also reducing the productive quality of farm animals used as food. Despite the known consequences of xenobiotic exposures, the current knowledge on xenobiotics, specifically heavy metals with regards to their diversity, toxicity mechanisms, and exposure-health risk relationships remain inadequate to inform regulatory and policy review necessary for managing environmental and occupational exposures. Therefore, this thematic topic was informed by the knowledge gap on metals and metal-induced toxicity.

Metals are ubiquitously distributed in the environment therefore humans and animals are unavoidably exposed. A study emphasized how arsenic exposure impairs a number of sensory systems in the body, therefore the need to consider arsenic exposure as a major public health concern that should warrant strict monitoring in the environment and work place. Corroboratively, urinary arsenic levels were linked to shifts in hearing threshold of adults in the United States (Long et al.). A study investigated environmental cadmium and the risk of kidney functional impairment and observed that cadmium exposure increased the risk of kidney stones (Ren et al.). Similarly, cadmium and polystyrene microplastics, two ubiquitous environmental contaminants synergistically upon co-exposure impair autophagic flux to increase lipid accumulation (Chen et al.) and these observations emphasize the need for

minimizing cadmium exposures at the workplace as well as in other exposure routes. Dimethylacetamide (DMAC) find application in the dye, leather and paint industries as a solvent. Diaminodiphenyl ether (4,4'-ODA), a precursor for synthesis of curing agents and dyes is a known inhibitor of the methemoglobin reduction system. A pipe leak from a plant containing DMAC and trace concentrations of 4,4'-ODA was linked to serious clinical toxicity (respiratory failure, methemoglobinemia, hemolytic anemia, and liver injury) in two exposed people in 2024. Although the two exposed people were treated and discharged, later one was diagnosed with encephalopathy 16 days post-exposure while the other developed peripheral neuropathy 58 days post-exposure (Zhang et al.). Metal exposures do not discriminate and in this light farm animals were not left out. A study assessed heavy metal exposure in farm animals and how it impacts on the reproductive and productive performance of livestock. Interestingly, it was observed that heavy metal exposure to livestock negatively impacted on reproductive and productive performance of the livestock raising concern on the long-term health consequences for humans who consume animal products from such exposed livestock (Afzal and Mahreen).

Treatment-related toxicity has been a public health concern, particularly use of some drugs in the management of human diseases. Xiong et al. studied the adverse effects associated with the use of sunitinib and ripretinib in the treatment of gastrointestinal stromal tumors analyzing data from the FDA adverse event reporting system (FAERS). The COVID-19 pandemic exposed the vulnerability of global emergency health preparedness and ever since efforts have been made to contain the COVID-19 pandemic. A key effort hovered around the avalanche of antiviral agents that were produced in record time to mitigate COVID-19. One of such antiviral agents was Azvudine. Hepatotoxicity was strongly associated with Azvudine (Xiong et al.). This observation may limit the clinical use of Azvudine particularly in COVID-19 patients with existing liver disease. It is instructive from this study to consider adding Azvudine to the Therapeutic Drug Monitoring (TDM) list in order to reduce toxicity while optimizing Azvudine use.

Mechanistic elucidation of metal poisoning is crucial to inform therapy. Guo et al. demonstrated mechanisms underlying cadmium and polystyrene induced hepatotoxicity upon co-exposure. Carnitine palmitoyl transferase I (CPT1) was implicated in cadmium and polystyrene microplastics co-exposure-induced hepatotoxicity in C57BL/6 mice and AML12 cells. Impaired copper homeostasis was shown to trigger cuproptosis in heart failure by impairing mitochondrial function (Liu et al.). Monocrotaline (MCT), a major pyrrolizidine alkaloid, is a known hepatotoxin. MCT induced autophagy in hepatocytes by inhibiting PI3K/AKT/mTOR signaling pathway (Guo et al.). Cantharidin (CTD), derived from *Mylabris*, a Chinese traditional medicine demonstrated anti-tumor effects but caused hepatotoxicity mediated through DDIT4/mTOR signaling pathway (Tang et al.).

Exploring novel remedies for metal poisoning is crucial in the present context of widespread metal exposures due to industrialization and technological advancement. Sentinel selenium doses were used to improve cadmium and lead induced decrease in hand grip strength (Liang et al.). Similarly, naringenin ameliorated cadmium chloride-induced renal injury by modulating autophagy, oxidative stress and endoplasmic reticulum stress (Shi et al.).

Collectively, all the studies presented in this thematic topic suggest the urgent need for elaborate understanding of exposure dynamics of heavy metals, mechanistic insights into heavy metal exposure-toxicity relationships. Establishment of exposure route-specific and soft tissue-specific limits for these heavy metals may be crucial in reducing health risk associated with these ubiquitously distributed toxicants. Health policymakers and regulatory agencies in the environmental chemical control space need to review existing policies and regulations. For instance, mandatory regular monitoring of heavy metals in vulnerable human populations as well as in matrices such as air, water, food products, and soil in order to prevent long term health risk upon exposure.

Author contributions

AB: Conceptualization, Writing – original draft, Writing – review and editing. YZ: Conceptualization, Resources, Supervision, Writing – review and editing. XL: Resources, Supervision, Writing – review and editing. MM: Conceptualization, Supervision, Writing – review and editing. FE: Conceptualization, Supervision, Writing – review and editing. XY: Supervision, Writing – review and editing.

Funding

The author(s) declare that no financial support was received for the research and/or publication of this article.

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OPEN ACCESS

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RECEIVED 19 October 2023

ACCEPTED 05 December 2023

PUBLISHED 14 December 2023

CITATION

Liang Y, Wang J, Wang T, Li H, Yin C, Liu J,
Wei Y, Fan J, Feng S and Zhai S (2023),
Moderate selenium mitigates hand grip
strength impairment associated with
elevated blood cadmium and lead levels
in middle-aged and elderly individuals:
insights from NHANES 2011–2014.
Front. Pharmacol. 14:1324583.
doi: 10.3389/fphar.2023.1324583

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Moderate selenium mitigates hand grip strength impairment associated with elevated blood cadmium and lead levels in middle-aged and elderly individuals: insights from NHANES 2011–2014

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Background: Selenium (Se) has been reported to have an antagonistic effect on heavy metals in animals. Nevertheless, there is a lack of epidemiological research examining whether Se can mitigate the adverse effects of cadmium (Cd) and lead (Pb) on hand grip strength (HGS) in middle-aged and elderly individuals.

Methods: This study used data from the 2011–2014 National Health and Nutrition Examination Survey (NHANES). HGS measurements were conducted by trained examiners with a dynamometer. Concentrations of Se, Cd, and Pb in blood were determined *via* inductively coupled plasma mass spectrometry. We employed linear regression, restricted cubic splines, and quantile g-computation (qgcomp) to assess individual and combined associations between heavy metals and HGS. The study also explored the potential influence of Se on these associations.

Results: In both individual metal and multi-metal models adjusted for confounders, general linear regression showed Se's positive association with HGS, while Cd and Pb inversely related to it. At varying Se-Cd and Se-Pb concentrations, high Se relative to low Se can attenuate Cd and Pb's HGS impact. An inverted U-shaped correlation exists between Se and both maximum and combined HGS, with Se's benefit plateauing beyond approximately 200 µg/L. Stratified analysis by Se quartiles reveals Cd and Pb's adverse HGS effects diminishing as Se levels increase. Qgcomp regression analysis detected Se alleviating HGS damage from combined Cd and Pb exposure. Subsequent subgroup analyses identified the sensitivity of women, the elderly, and those at risk of diabetes to HGS impairment caused by heavy metals, with moderate Se supplementation beneficial in mitigating this effect. In the population

at risk for diabetes, the protective role of Se against heavy metal toxicity-induced HGS reduction is inhibited, suggesting that diabetic individuals should particularly avoid heavy metal-induced handgrip impairment.

Conclusion: Blood Cd and Pb levels are negatively correlated with HGS. Se can mitigate this negative impact, but its effectiveness plateaus beyond 200 µg/L. Women, the elderly, and those at risk of diabetes are more vulnerable to HGS damage from heavy metals. While Se supplementation can help, its protective effect is limited in high diabetes risk groups.

KEYWORDS

heavy metals, toxicity, selenium, cadmium, lead, hand grip strength

1 Introduction

Selenium (Se) is an essential trace element present in various dietary sources, including grains, nuts, vegetables, and different animal products (Fan et al., 2023). It holds pivotal roles in numerous health aspects, including its antioxidant properties (Li et al., 2017), bolstering the immune system (Razaghi et al., 2021), tumor prevention (Razaghi et al., 2021), maintaining cardiovascular health (Mohammadifard et al., 2019), and enhancing renal function (Wang et al., 2022). Yet, the balance of Se intake is crucial; while deficiency can be harmful, excessive intake can also pose health risks. Supplementation of Se has been linked with enhanced immune responses, particularly during pregnancy, autoimmune thyroid disorders, and post-severe illnesses (Schomburg, 2021). On the flip side, high Se levels might impair neurological functions and increase the risk of type 2 diabetes (Genchi et al., 2023). Intriguingly, a Mendelian randomized study suggested that high Se levels might be detrimental to renal function (Fu et al., 2022). However, in contrast, Se supplementation has shown promise in improving kidney function in individuals with acute kidney injury or chronic kidney diseases (Iglesias et al., 2013). This underscores a dose-response relationship concerning Se's health effects. Furthermore, a study highlighted that combined supplementation of zinc (Zn) and Se could boost physical functionality in overweight individuals undergoing calorie-restricted diets, hinting at Se's potential benefits in enhancing physical performance (Zavros et al., 2023).

Heavy metals, in contrast to trace elements, pose health hazards regardless of their concentration. Persistent exposure to these toxic elements entails significant health challenges. Abundant research delineates that such exposures can catalyze a gamut of health complications, from cardiovascular ailments and cancers to diabetes and renal dysfunctions, etc (Yao et al., 2021). Two commonly encountered heavy metal contaminants, lead (Pb) and cadmium (Cd), amass in human systems owing to the biomagnification phenomenon in the food chain (Li et al., 2023). Pb exerts profound detrimental effects on the brain, injuring its vascular endothelium, leading to cerebral microvessel dysfunction. This can cause aberrations in cerebral blood circulation, enhancing the likelihood of intracranial carotid artery atherosclerosis, which in turn, raises the potential for cardiovascular complications or strokes (Lin et al., 2018). An assessment centered on elderly individuals unveiled a negative correlation between Cd exposure and Hand grip strength (HGS) (García-Esquinas and Rodríguez-Artalejo, 2017). In a parallel vein, research deduced that amplified Pb exposure

adversely influenced grip strength, alluding to a probable linkage between pediatric HGS and heavy metal pollutants (Wu et al., 2022). Animal experiments indicate that the toxic effects of Pb on muscles primarily manifest in disrupting normal glucose metabolism processes, altering the activity of key enzymes, and adjusting energy metabolism, which may also damage muscle tissue structure. These physiological changes could negatively impact muscle function and quality, thereby adversely affecting overall health (Das et al., 2020). In another animal study, it was found that Pb exposure's harm to skeletal muscle function mainly includes reduced muscle mass, decreased exercise capacity, weakened muscle strength, disrupted lipid metabolism, increased inflammatory responses, and upregulated expression of pro-apoptotic genes. These damages are likely due to lead's direct effects on muscle cell structure, metabolism, and inflammatory pathways (He et al., 2023). Further research also shows that Cd's damage to skeletal muscles mainly manifests as reduced muscle weight and strength, decreased exercise capability, disrupted lipid metabolism, intensified inflammatory responses, and enhanced expression of apoptotic genes. These injuries might originate from Cd's direct toxic action on muscle cells and the associated inflammatory reactions (He et al., 2023). On the other hand, considering that the critical values for grip strength diagnostic criteria differ by gender, and the physiological differences between men and women, the impact of heavy metals on male and female grip strength may exhibit significant gender differences. However, research on the gender differences in the effects of heavy metals on male and female grip strength remains relatively insufficient. Existing literature suggests that men and women differ in risk factors associated with reduced grip strength (Sternäng et al., 2015). Studies have found that early childhood Pb exposure is associated with loss of brain structural volume, and this Pb exposure-related brain volume reduction is more pronounced in male participants (Cecil et al., 2008). Research on African Americans reveals significant lifetime Pb content differences, with women potentially facing higher health risks due to Pb release from bones (Theppeang et al., 2008). Additionally, the potential impact of Cd exposure on cardiovascular health indicators shows a statistically significant association in Caucasian men and Mexican-American women (Egwuogu et al., 2009). Given these ramifications, tackling the consequences of heavy metal exposure emerges as a paramount public health imperative.

HGS gauges an individual's ability to grasp objects, reflecting the force exerted by hand muscles. A prevalent tool in clinical environments, HGS serves as an indicator of hand strength and

overall muscle functionality (Wang et al., 2018). From a physiological standpoint, HGS typically peaks in early adulthood, maintains stability through midlife, and then gradually declines, predominantly impacting those in middle age and beyond (Dodds et al., 2014). Numerous clinical and epidemiological studies highlight HGS's predictive value, associating it with both short-term and long-term health outcomes (Sayer and Kirkwood, 2015). In clinical contexts, a decreased HGS can signal potential post-operative complications, prolonged hospital stays, increased rates of hospital readmission, and general physical decline. Among the elderly, reduced HGS often corresponds with a loss of independence (Labott et al., 2019). Furthermore, epidemiological data suggest that even in healthy adults, a declining HGS can foretell forthcoming functional limitations, disabilities in old age, and an elevated risk of mortality (Norman et al., 2011). Given these implications, bolstering HGS stands out as a vital health strategy, especially for the middle-aged and elderly populations.

In essence, while moderate intake of Se offers health benefits, toxic heavy metals adversely impact HGS. This presents a critical query: Can Se counterbalance the detrimental effects of heavy metals, potentially safeguarding HGS by tempering their toxicity? Several experimental studies suggest that Se element could mitigate the negative impacts of heavy metals on animal cognition (Ren et al., 2022). A population-based study inferred that moderate Se intake might counter the lung function deterioration induced by Pb and Cd in humans (Fan et al., 2023). Another research centered on the elderly in China proposed that Se might neutralize the harmful effects of Pb and Cd on cognitive functions (Cheng et al., 2023). Consequently, it is conceivable that an optimal dose of Se can potentially counteract the damaging effects of heavy metal exposure. However, to translate this hypothesis into actionable clinical recommendations, it needs more expansive epidemiological research and rigorous clinical trials.

Given the existing research landscape, there's a noticeable gap in understanding whether Se can counteract the decline in HGS due to heavy metals, especially Pb and Cd. Addressing this void, our study utilized data from the National Health and Nutrition Examination Survey (NHANES) to meticulously explore the relationships between blood concentrations of Se, Cd, and Pb and their collective impact on HGS. The insights from this study aim to broaden our understanding of how optimal Se intake might counterbalance the adverse effects of Cd and Pb on HGS. This knowledge could inform suitable Se supplementation recommendations and pave the way for pioneering therapeutic approaches to counteract HGS decline attributed to heavy metal toxicity.

2 Methods

2.1 Study population

In this study, we utilized data from the NHANES, which is designed to gather data emblematic of the civilian, non-institutionalized US population, aiming to discern the prevalence of significant diseases and pinpoint their associated risk factors. The datasets employed for this research were extracted from two NHANES survey cycles: 2011–2012 and 2013–2014. In our cross-

sectional analysis, certain participants were omitted based on the following criteria: 1) individuals aged below 45, accounting for 13,524 participants; 2) those with absent data on Se, Pb, and Cd, totaling 2,047 participants; and 3) participants lacking handgrip test data, comprising 518 individuals.

Following these exclusions, this analysis encompassed 3,842 participants, primarily from the middle-aged and elderly demographics.

2.2 Assessment of HGS

The muscle strength/grip test component evaluated isometric HGS using a handgrip dynamometer. The grip test was conducted in a standing position unless the participant had physical limitations. This study measured participants' muscle strength in two ways: maximum HGS and combined HGS. Participants performed the HGS test three times for both left and right hands. The study took the highest value out of the three tests as the participant's maximum HGS; the combined HGS refers to the sum of the largest reading from each hand.

2.3 Assessment of Se, Cd, and Pb

Three elements of toxicological and nutritional interest including Cd, Pb, and Se in whole human blood specimens are processed, stored, and later shipped to the National Center for Environmental Health and the Centers for Disease Control and Prevention for test, which were measured directly using mass spectrometry after a simple dilution sample preparation step. Optimal amount of specimen is 1–2 mL. Request a minimum volume of 0.4 mL. Volume for one analytical measurement is 0.1 mL. No fasting or special diets are required before collection of blood. The lower detection limits for Pb were 0.25 µg/dL, 0.16 µg/L for Cd and 30 µg/L for Se, respectively. It is imperative to mention that values falling beneath the established LLODs for Pb, Cd, and Se are computed as half the LLOD value squared. All participants have signed the informed consent. More details about the whole blood metals can be seen in [Supplementary Material](#) (1.4 Supplementary Explanation on the Detailed Measurement Process of Se, Cd, and Lead).

2.4 Covariates

For this analysis, participants' sociodemographic details, lifestyle attributes, and additional body metrics were considered. The sociodemographic data primarily captured age, sex, race (categorized into Mexican American, non-Hispanic white, non-Hispanic African American, other Hispanic, and other races), marital status (distinguished as married or unmarried), and educational level (delineated as having a bachelor's degree or higher, or below). Lifestyle parameters encompassed aspects like alcohol intake (quantified by consuming a minimum of 12 alcoholic beverages annually), smoking habits (with serum cotinine levels serving as a nicotine exposure marker to evaluate smoke exposure), and body mass index (BMI). Other pertinent body metrics integrated into the study were systolic blood pressure (SBP), diastolic blood pressure (DBP), HbA1c levels, total cholesterol

TABLE 1 Characteristics in participants stratified by the blood Se quartile.

Characteristics	Overall (n = 3,842)	Q1 (n = 961)	Q2 (n = 960)	Q3 (n = 960)	Q4 (n = 961)	p-value
Age, (year)	61.78 ± 10.65	63.16 ± 11.23	61.71 ± 10.65	61.2 ± 10.33	60.9 ± 10.24	<0.001
Gender, n (%)						<0.001
Female, n (%)	1964 ± 51.1	524 ± 54.5	533 ± 55.5	477 ± 49.7	430 ± 44.7	
Male, n (%)	1860 (48.9)	437 (45.5)	427 (44.5)	483 (50.3)	531 (55.3)	
Race, n (%)						<0.001
Mexican American	344 (9.0)	69 (7.2)	96 (10.0)	93 (9.7)	86 (8.9)	
Non-Hispanic white	383 (10.0)	110 (11.4)	89 (9.3)	103 (10.7)	81 (8.4)	
Non-Hispanic African American	1652 (43.0)	416 (43.3)	412 (42.9)	395 (41.1)	429 (44.)6	
Other Hispanic	965 (25.1)	283 (29.4)	246 (25.6)	241 (25.1)	195 (20.)3	
Other races	498 (13.0)	83 (8.6)	117 (12.2)	128 (13.3)	170 (17.)7	
College or above, n (%)	1998 ± 52.0	424 ± 44.2	486 ± 50.6	539 ± 56.2	549 ± 57.1	<0.001
Married, n (%)	2,252 ± 58.6	478 ± 49.7	562 ± 58.6	608 ± 63.3	604 ± 62.9	<0.001
Drinking, n (%)	2,559 ± 70.9	598 ± 67.5	628 ± 69.9	673 ± 73.1	660 ± 72.9	0.025
Cotinine, (ng/mL)	55.24 ± 135.24	71.57 ± 141.20	54.06 ± 124.94	43.24 ± 121.39	52.16 ± 149.89	<0.001
SBP, (mmHg)	128.87 ± 18.56	129.88 ± 20.51	128.95 ± 18.86	128.39 ± 17.75	128.27 ± 16.92	0.255
DBP, (mmHg)	70.63 ± 13.45	69.29 ± 13.82	70.68 ± 13.10	71.10 ± 13.97	71.45 ± 12.81	0.005
TC, (mg/dL)	196.22 ± 43.52	186.74 ± 40.61	195.03 ± 41.81	199.64 ± 44.20	203.33 ± 45.53	<0.001
BMI, (kg/m ²)	29.31 ± 6.73	29.28 ± 7.38	29.52 ± 6.74	29.37 ± 6.58	29.06 ± 6.18	0.492
HbA1c, (%)	6.00 ± 1.16	5.93 ± 0.99	5.94 ± 1.08	6.00 ± 1.11	6.13 ± 1.39	<0.001
Max HGS, (Kg)	32.8 ± 10.33	30.9 ± 10.21	32.5 ± 10.36	33.6 ± 10.22	34.2 ± 10.22	<0.001
Combined HGS, (Kg)	65.6 ± 20.69	61.6 ± 20.35	65.0 ± 20.61	67.3 ± 20.66	68.4 ± 20.50	<0.001

Note: Percentages were computed including those with missing values. Data are expressed as numbers (percentages) for categorical variables and as w mean (standard deviations) for continuous variables; Q1: Se < 178 µg/L; Q2: 178–192 µg/L; Q3: 192–208 µg/L; Q4: Se ≥ 208 µg/L.
Abbreviation: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; HbA1c, hemoglobin A1c; HGS, hand grip strength.

(TC), the metabolic equivalent of task (MET)score and HEI-2015 index. The specific details of covariates can be found in [Supplementary Table SA3](#).

2.5 Statistical analysis

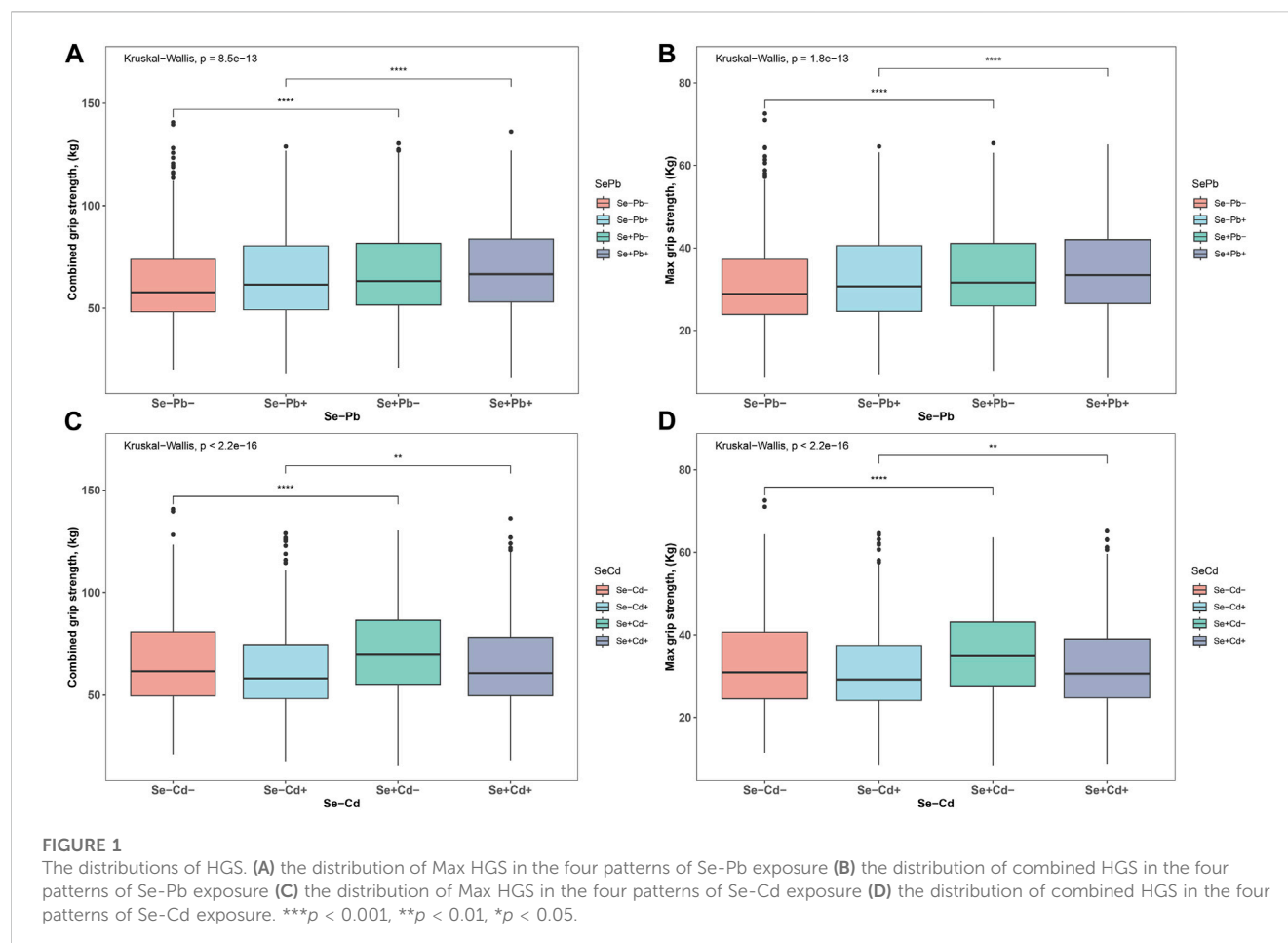
In this study, continuous variables are presented as mean ± SD, while categorical variables are described by their frequencies and percentages. Blood concentrations of heavy metals are represented by the median with the inter-quartile range (IQR). Depending on the data type, group characteristics were compared using the chi-square test, ANOVA, or the Kruskal–Wallis H-test. To investigate the relationship between exposure and HGS, we employed both univariate and multivariate linear regression models. These included an unadjusted model, a fully adjusted version, and a multi-metal model designed to account for confounders due to heavy metals. We further examined the effect of Se on the association between blood levels of heavy metals (Cd and Pb) and HGS by using multivariable linear regressions and restricted cubic splines based on blood Se quartiles. Additionally, the study explored the associations between four specific Se-Cd or Se-Pb

exposure patterns and HGS. The quantile g-computation model was utilized to precisely determine the relationships between mixed heavy metal exposures and HGS. All statistical analyses were performed with R software (version 4.3.1). A two-tailed *p*-value of less than 0.05 was considered statistically significant. In the subgroup analysis, this study categorized participants into gender subgroups (male and female) and age subgroups (<60, ≥60). The results of the subgroup analysis are presented in [Supplementary Tables S7–S8](#). In the sensitivity analysis, this study conducted additional adjustments for two variables that could potentially influence HGS levels, namely, MET score and HEI-2015. Detailed calculation methods and descriptions can be found in [Supplementary Materials](#) Section 1.1 and Section 1.2.

3 Results

3.1 Characteristics of selected population

[Table 1](#) provides a detailed overview of the primary attributes of both the general population and the study’s participants, segmented according to Se quartiles from the NHANES dataset spanning



2011–2014. The cohort included 3,842 participants, displaying a near-even gender distribution: 48.9% male and 51.1% female, with an average age of 61.78 ± 10.65 years. Notably, individuals in the higher Se quartiles exhibited distinct features: they were generally younger, predominantly male, had a higher representation of non-Hispanic African Americans, exhibited greater academic achievements, were more frequently married, and presented a larger portion with lower BMI values. In contrast, participants with lower Se concentrations were more inclined towards smoking and alcohol consumption and had elevated blood pressure, TC, and HbA1c levels. A comprehensive overview of baseline attributes related to Se, Pb, and Cd can be found in [Supplementary Table S1](#).

The study's results emphasize a significant positive correlation between maximum grip strength and Se concentrations. As Se levels increased, there was a marked improvement in maximum grip strength. This correlation was evident across quartiles, with measurements as follows: Quartile 1 averaged 30.9 ± 10.21 kg, Quartile 2 averaged 32.5 ± 10.36 kg, Quartile 3 averaged 33.6 ± 10.22 kg, and Quartile 4 averaged 34.2 ± 10.22 kg ($p < 0.001$). Similarly, the combined grip strength demonstrated a consistent trend across quartiles: Quartile 1 at 61.6 ± 20.35 kg, Quartile 2 at 65.0 ± 20.61 kg, Quartile 3 at 67.3 ± 20.66 kg, and Quartile 4 at 68.4 ± 20.50 kg ($p < 0.001$).

Figure 1 provides a comprehensive distribution analysis of both maximum and combined HGS across two primary exposure combinations: Cd-Se and Pb-Se. When assessing maximum HGS,

the groups with Se presence, regardless of Cd or Pb exposure, consistently showcased superior HGS outcomes compared to their counterparts without Se (all $p < 0.001$). Specifically, the Se + Cd-group notably outperformed the Se- Cd-group, and similar patterns were observed for the Pb exposure groups. Turning attention to combined HGS, the presence of Se again emerged as a beneficial factor, enhancing grip strength across both Cd and Pb exposures. Notably, the Se + Cd-group demonstrated a heightened combined HGS over the Se- Cd-group, with similar superior results observed for the Se + groups in Pb exposure scenarios (all $p < 0.001$). Collectively, these findings underscore the potential protective role of Se in counteracting the detrimental impacts of both Cd and Pb on HGS.

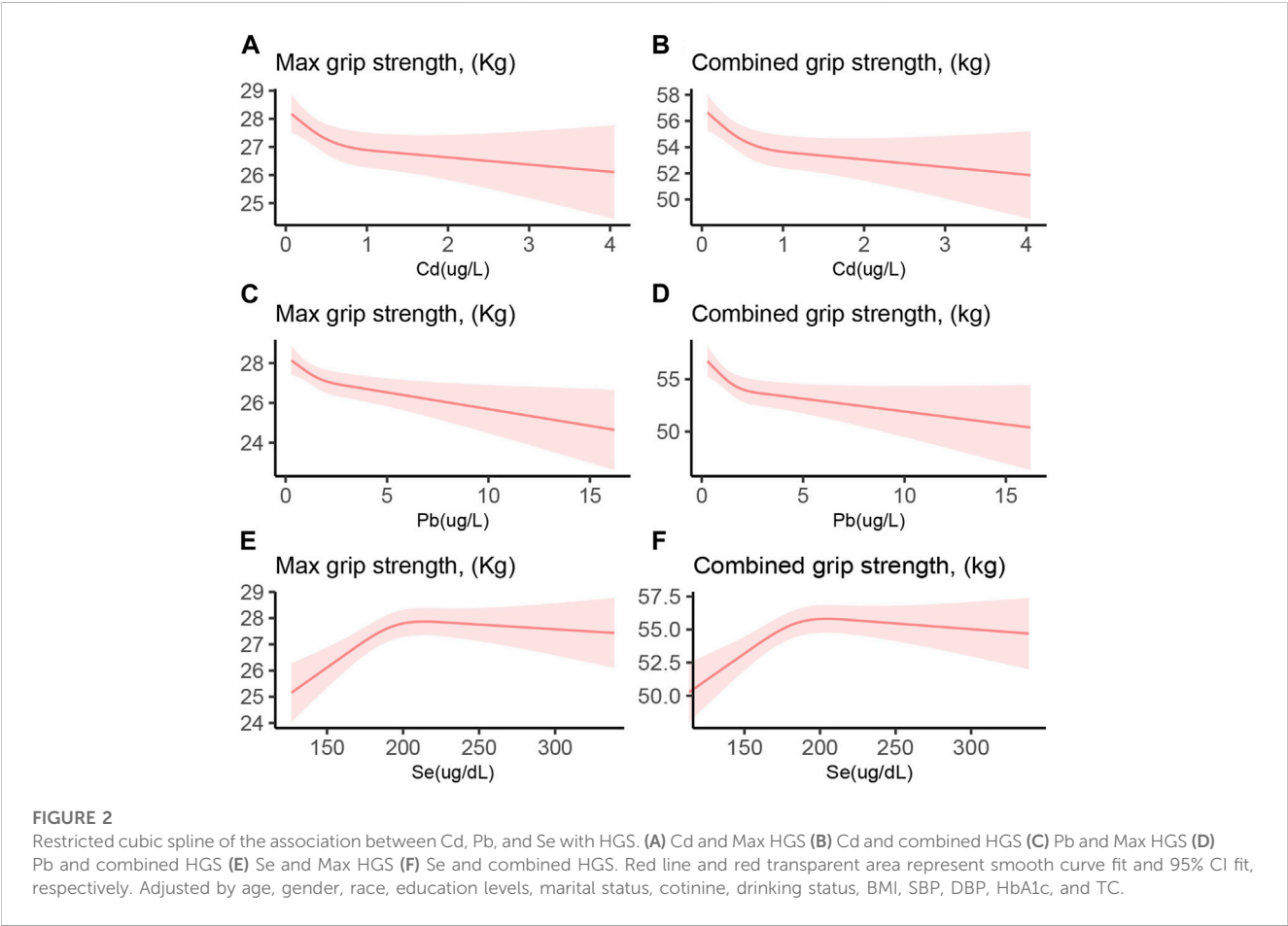
3.2 Association of individual metals with HGS

Table 2 summarizes the results from a general linear regression analysis examining the associations between Cd, Pb, and Se levels with HGS. In the unadjusted single-metal model, Cd was negatively correlated with HGS, while both Pb and Se demonstrated positive correlations with HGS. In the adjusted single-metal model, Cd retained its negative association with HGS, and Se continued to correlate positively. However, the relationship between Pb and HGS became negative. In the adjusted multi-metal model, the negative association of Cd with HGS persisted (maximum: $\beta = -0.45$, CI

TABLE 2 Associations between Cd, Pb, Se and HGS using general linear regression.

Outcomes	Single metal (unadjusted)		Single metal (adjusted) ^a		Multiple metals (adjusted) ^b	
	β (95% CI)	p-value	β (95% CI)	p-value	β (95% CI)	p-value
Max HGS						
Cd	−0.71 (−1.26, −0.18)	0.008	−0.56 (−0.98, −0.15)	0.008	−0.45 (−0.87, −0.04)	0.033
Pb	0.44 (0.27, 0.63)	<0.001	−0.02 (−0.35, −0.10)	<0.001	−0.20 (−0.33, −0.08)	0.002
Se	0.03 (0.02, 0.04)	<0.001	0.01 (0.00, 0.02)	0.006	0.01 (0.00, 0.02)	0.010
Combined HGS						
Cd	−1.58 (−2.65, −0.50)	0.004	−1.29 (−2.13, −0.46)	0.002	−1.10 (−1.94, −0.26)	0.006
Pb	0.89 (0.53, 1.26)	<0.001	−0.41 (−0.66, −0.16)	0.001	−0.35 (−0.61, −0.11)	0.010
Se	0.07 (0.04, 0.09)	<0.001	0.02 (0.00, 0.03)	<0.001	0.02 (0.00, 0.03)	0.011

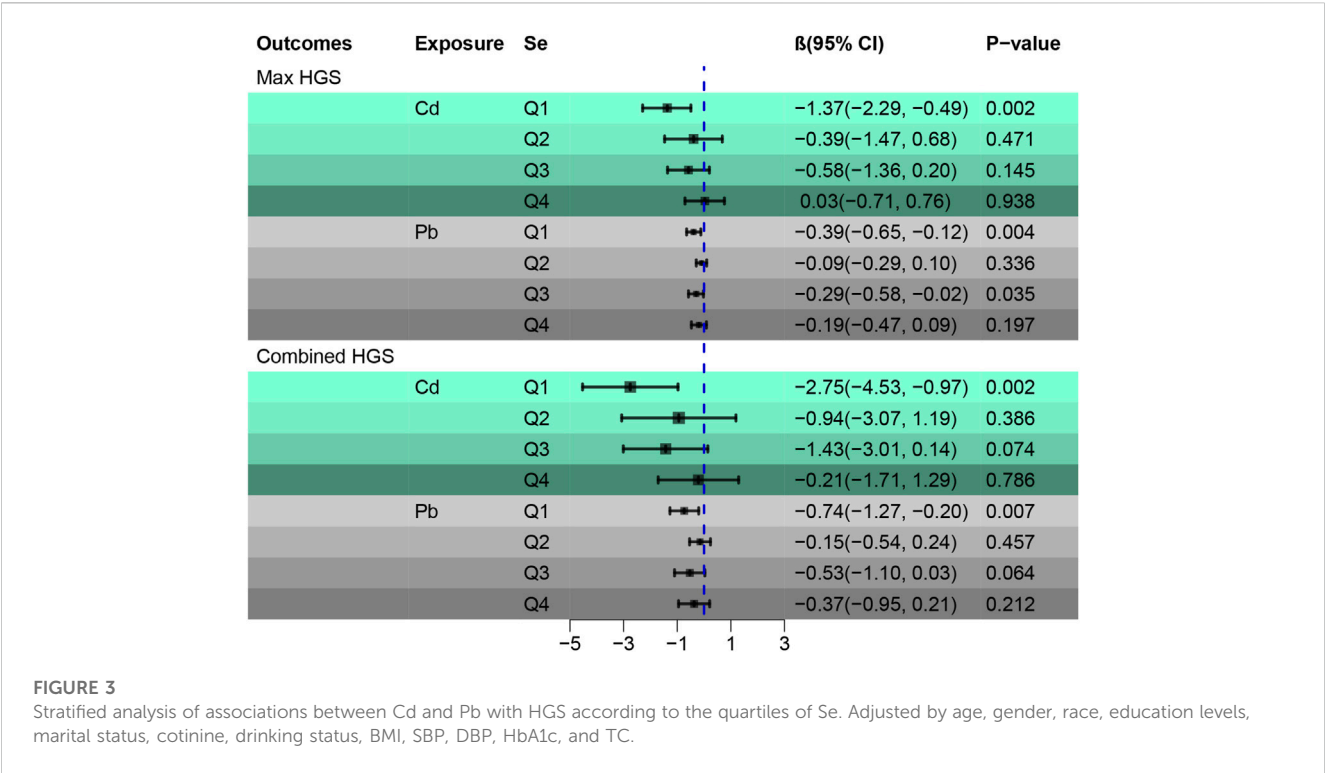
^aCovariates in adjusted single-metal models included age, gender, race, education levels, marital status, cotinine, drinking status, BMI, SBP, DBP, HbA1c, TC.
^bCovariates in multiple-metal models included covariates in adjusted single-metal models and the other two metals except for the independent variable.



[−0.87, −0.04]; combined: $\beta = -1.10$, CI [−1.94, −0.26]). Pb exhibited a negative relationship with HGS (maximum: $\beta = -0.20$, CI [−0.33, −0.08]; combined: $\beta = -0.35$, CI [−0.61, −0.11]), while the positive association of Se with HGS remained consistent (maximum: $\beta = 0.01$, CI [0.00, 0.02]; combined: $\beta = 0.02$, CI [0.00, 0.03]).

Figure 2 depicts the relationship between the blood concentrations of Cd, Pb, and Se and HGS. A blood Se

concentration exceeding approximately 200 $\mu\text{g/L}$ does not further enhance HGS, demonstrating an inverted U-shaped relationship. Conversely, an increase in blood Pb concentration corresponded with a decline in HGS, indicating a direct inverse relationship. [Supplementary Table S2](#) categorizes Se concentrations into four quartiles. Compared to the quartile with the lowest Se levels, the second quartile exhibited significantly enhanced HGS.



However, the third and fourth quartiles did not exhibit comparable strength. This observation implies that excessively high Se concentrations may be detrimental to HGS.

3.3 Role of Se on the association between heavy metals (Cd and Pb) and HGS

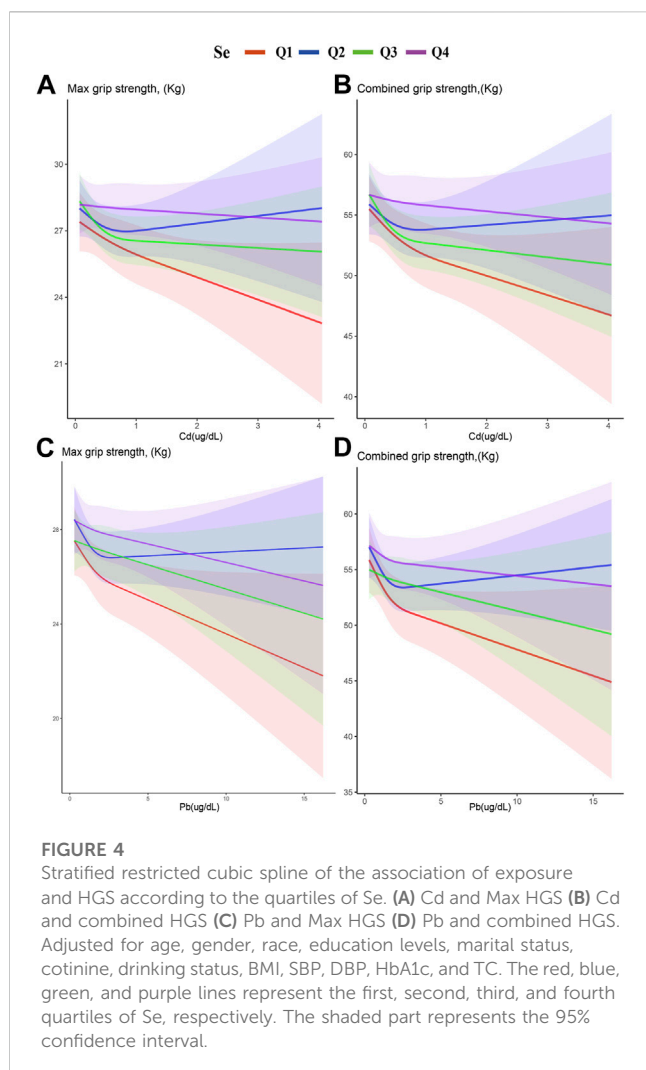
Figure 3 provides a stratified analysis based on Se quartiles, examining the associations between Cd and Pb with HGS. It elucidates the interplay between blood concentrations of Cd and Pb with HGS across varying Se concentrations. For Max HGS, the deleterious impacts of Cd and Pb are attenuated with increasing Se concentrations. Significantly, participants within the second and fourth Se quartiles experienced the least harm from Cd on Max HGS. Similarly, the negative influence of Pb on Max HGS was substantially reduced within these Se quartiles. With regard to combined HGS, a consistent trend is observed: the adverse effects of both Cd and Pb recede as Se levels increase. Specifically, the second and fourth Se quartiles showcased participants least affected by Cd and Pb concerning combined HGS. For more detailed data, please refer to [Supplementary Table S3](#).

[Supplementary Table S4](#), using a general linear regression, delves deeper into the relationship between Se-Cd and Se-Pb exposures and grip strength. Taking the Se-Cd-group as a baseline, the β value for the Se + Cd + group falls between that of the Se + Cd- and Se- Cd + groups. This pattern implies that Se mitigates the detrimental impacts of Cd on Max HGS. An analogous pattern is discerned for Se-Pb, underscoring Se's protective effect against the harmful influences of both Cd and Pb on HGS. These findings underscore the significant interplay between Se, Cd, and Pb, emphasizing Se's potential protection role on HGS.

Figure 4 displays restricted cubic splines, stratified by Se quartiles. The negative correlation between Cd and Pb with HGS weakens by ascending Se levels. This attenuation is most notable between the first and second Se quartiles, where the second quartile shows the weakest correlation. Intriguingly, the fourth Se quartile reveals a resurgence of the inverse relationship between Cd and Pb with HGS. This pattern suggests that there exists an optimal range of Se concentrations wherein it most effectively mitigates the adverse impacts of Cd and Pb on HGS.

Figure 5 Employs the Qgcomp regression analysis to examine the relationship between mixed metal exposures (Cd, Pb) in the blood and HGS across various Se quartiles. A pronounced negative association was found between Cd and Pb mixtures with both Max ($\beta = -0.68$, 95% CI [-0.96, -0.40]) and combined HGS ($\beta = -1.51$, 95% CI [-2.07, -0.95]), establishing statistical significance ($p < 0.001$). Stratified Qgcomp regression reveals that the adverse effects of heavy metals on Max HGS are somewhat buffered when Se concentrations lie within the second and third quartiles, compared to the more pronounced effects observed in the first and fourth quartiles. This pattern indicates Se's capacity to counteract the detrimental impacts of heavy metals on Max HGS. A similar trend was discerned for combined HGS. For more detailed data, please refer to [Supplementary Table S5](#).

Moreover, [Supplementary Table S6](#) delves into the correlations between a broader metal mixture in the blood (Se, Cd, Pb) and grip strength. Notably, apart from the observed association with Max HGS ($\beta = -0.83$, 95% CI [-1.53, -0.14]), other correlations did not reach statistical significance. Collectively, these insights emphasize Se's potential protective role against the adverse influences of heavy metal exposures on HGS.



3.4 Subgroup analyses

In the subgroup analysis, additional adjustments were made for the “MET score and HEI-2015 index.” The detailed description of adjusted variables is provided in [Supplementary Tables SA1, SA2](#).

The Qgcomp regression method examines the relationship between mixed blood metals (Cd and Pb) across different Se quartiles in relation to HGS. This method effectively stratifies, and groups data based on gender and age.

[Supplementary Table S7](#) highlights gender-specific disparities. The composite of Cd and Pb shows a negative correlation with both Max and combined HGS. This negative association is more discernible among females. As Se levels rise, its protective effect against heavy metal-induced HGS impairment in females exhibits an initial strengthening followed by a subsequent weakening trend. In contrast, for males, a non-linear positive trend emerges, though it lacks statistical significance. These observations suggest that females might be more vulnerable to heavy metal-induced impairment in grip strength, and specific Se concentrations can counteract such effects.

[Supplementary Table S8](#) dissects age-related variations. The combined effects of Cd and Pb are inversely correlated with both Max and combined HGS. However, this correlation is notably stronger among older individuals, suggesting a more

pronounced detrimental impact of heavy metals on the elderly’s HGS. With the elevation of Se concentration, its capacity to mitigate the adverse effects of heavy metals on HGS in the elderly follows an initial strengthening followed by a subsequent weakening trend. Conversely, a non-linear positive trend emerges in the middle-aged group, although it does not achieve statistical significance. These findings emphasize the heightened susceptibility of the elderly to HGS reductions due to heavy metal toxicity and the protective role of Se within a specific concentration range.

[Supplementary Table S9](#) detailed the analysis related to diabetes, categorizing participants into two groups based on glycated hemoglobin (HbA1c) levels: at risk of diabetes ($\text{HbA1c} \geq 6.5$) and non-diabetic ($\text{HbA1c} < 6.5$). The study found that the combined effects of Cd and Pb on maximal and overall HGS were negatively correlated in both groups. However, a deeper stratified analysis using qgcomp regression revealed that in the diabetes risk group, only the lowest quartile (Q1) of Se (Se) showed a significant statistical correlation with HGS, while higher quartiles (Q2, Q3, Q4) did not show a significant association. In contrast, in the non-diabetic group, both Q1 and Q3 quartiles of Se were correlated with HGS, and a negative trend was observed across quartiles Q1 to Q4. These results underscore the limited protective role of Se in mitigating HGS decline caused by heavy metal toxicity in the diabetes risk group. Therefore, individuals at risk of diabetes should be particularly cautious about heavy metal-induced handgrip strength impairment. These findings provide important perspectives for future research, especially regarding the impact of heavy metal exposure on HGS in populations with different health statuses.

3.5 Sensitivity analyses

A sensitivity analysis was conducted wherein further adjustments were made for HGS-related variables. In the sensitivity analysis, additional adjustments were made for “MET score and HEI-2015 index.” This adjustment aimed to explore the relationship between both individual and combined blood metals (Cd and Pb) across different Se quartiles and HGS. This relationship was assessed using stratified analysis and the Qgcomp regression approach.

[Supplementary Table S10](#) reveals that as Se concentrations escalate, its potential to offset the adverse impacts of individual heavy metals on HGS emerges as a non-linear positive trend, though this trend does not reach statistical significance. Conversely, [Supplementary Table S11](#) indicates that the combination of Cd and Pb has an inverse relationship with both maximum and total grip strength. With increasing Se levels, its protective role against the negative effects of combined heavy metal exposure on HGS becomes evident, following a non-linear positive trajectory that is statistically significant.

4 Discussion

Environmental pollutants, especially heavy metals, severely impact human health. In this study, we observed a negative correlation between heavy metals in the blood, including Cd and

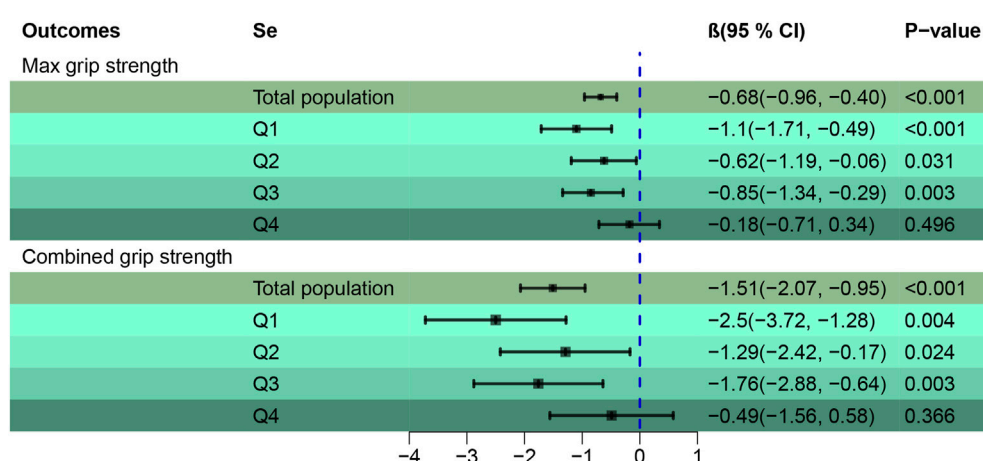


FIGURE 5

Qgcomp regression to assess the association of the mixture of blood metals (Cd, Pb) with HGS across Se quartiles. Adjusted by age, gender, race, education levels, marital status, cotinine, drinking status, BMI, SBP, DBP, HbA1c, and TC.

Pb with HGS, while Se showed a positive correlation with HGS. However, it is noteworthy that this positive correlation diminishes when the concentration of Se in the blood exceeds about 200 $\mu\text{g/L}$. Only moderate levels of Se can mitigate the adverse effects of Cd and Pb on HGS. Therefore, focusing on the impact of heavy metals on HGS not only helps in identifying high-risk exposures to heavy metals and reducing environmental contact with them but also emphasizes the importance of appropriately supplementing with the Se element. This research provides crucial information for reducing HGS damage due to heavy metal exposure. To the best of our understanding, we demonstrated that both the higher and lower blood Se concentrations were detrimental to the middle and elderly' HGS impairment related to Cd and Pb for the first time. Only moderate Se could mitigate the adverse effect of Cd and Pb on HGS.

4.1 Comparison with other studies

This study employed data from the NHANES spanning 2011 to 2014. It aimed to discern both individual and combined effects of Cd and Pb exposure levels in the blood on HGS among middle-aged and elderly. Additionally, the study probed the potential protective effect of Se supplementation on these relationships, shedding light on a new interplay between Se, Cd, and Pb, with HGS.

Historically, numerous studies have highlighted the health benefits of Se and the hazards of heavy metal toxicity. One study suggested that heightened Cd exposure corresponds with decreased HGS, underscoring the potentially harmful influence of heavy metals on HGS (García-Esquinas et al., 2020). Another study focusing on elderly women drew a correlation between low serum Se levels and diminished HGS, implying that Se supplementation might enhance HGS (Beck et al., 2007). However, investigations into Se's countering effect on health issues induced by heavy metal toxicity remain limited. A recent cross-sectional study postulates that optimal Se levels can counter the toxic effects of Cd and Pb, particularly on lung function (Fan et al., 2023). This intimates that Se might play a pivotal role in

mitigating health concerns stemming from heavy metal toxicity. The research findings align with our study's principles and outcomes, underscoring the impact of heavy metal exposure on HGS. Adequate Se levels may mitigate the toxicity of these heavy metals by enhancing the activity of certain detoxifying enzymes. This Se-mediated effect should not be perceived as a direct barrier but rather as a biochemical mechanism that combats the toxicity of heavy metals at the cellular level.

4.2 Potential mechanism

Pb and Cd, as toxic metals with significant occupational health implications, are playing an increasingly important role in current environmental pollution studies. The impact of these metals on organs and biological systems is widespread and profound, capable of causing serious health problems, including acute and especially chronic poisoning symptoms. A potential correlation exists between blood levels of Se, Cd, and Pb with HGS, potentially arising from various underlying mechanisms.

4.2.1 The toxic mechanisms of Cd and lead

Research findings reveal that Cd is an effective inducer of oxidative stress, DNA damage, endoplasmic reticulum (ER) stress, and autophagy. Excessive stress responses can lead to significant tissue damage by triggering various cellular death pathways such as apoptosis, ferroptosis, and necroptosis (Ma et al., 2022). Particularly, the kidneys, liver, bones, and nervous system, which are the main targets of chronic Cd exposure, are proven to be at increased risk of kidney damage (Barregard et al., 2022), liver injury (Bernhoft, 2013), neurodegenerative diseases (Ma et al., 2022), and osteoporosis (Luo et al., 2021) due to environmental and occupational Cd exposure. Similarly, environmental contamination with Pb is a global public health issue, associated with various diseases, especially neurodegenerative diseases, where microglia-mediated neuroinflammation plays a key role in lead's neurotoxicity (Hu

et al., 2023). Additionally, research indicates a negative correlation between Cd and Pb with HGS, suggesting that these heavy metals may impair HGS by affecting multiple physiological mechanisms.

The common mechanism of toxicity induced by heavy metals is believed to be due to their induction of excessive production of reactive oxygen species (ROS), depletion of antioxidants, and inhibition of antioxidant defense enzyme activities. This disrupts the cellular redox balance, leading to adverse health effects (Rehman et al., 2018; Balali-Mood et al., 2021). Epidemiological studies and laboratory data both indicate that heavy metal-induced oxidative stress is closely related to the cellular response to increased risks of tumors, neurological diseases, diabetes, infertility, developmental disorders, renal dysfunction, and cardiovascular diseases (Paithankar et al., 2021). In normal cellular metabolism, the production of reactive oxygen species (ROS) is regulated by the cellular antioxidant system to maintain a balanced state. The oxidative stress caused by Cd and Pb in the body, through inducing excessive production of ROS, leads to the imbalance of the cellular antioxidant regulatory system (Matović et al., 2015). Redox-inactive metals such as Cd, arsenic (As), and Pb exhibit their toxicity by binding to the sulfhydryl groups of proteins and depleting glutathione (Jomova and Valko, 2011; Jozefczak et al., 2012). Studies have found that increased blood Pb levels are associated with decreased serum levels of the anti-aging protein alpha-klotho (α -klotho), suggesting that Pb exposure may exacerbate oxidative stress by affecting the expression or activity of α -klotho, leading to aging manifestations like muscle loss, which may affect handgrip strength (Kim et al., 2022). Animal studies also show that Cd accumulation can lead to a decrease in the activity of antioxidant enzymes (such as T-SOD, GST, and GPX), causing myocardial inflammation (Guo et al., 2020).

Cellular toxicity caused by heavy metal exposure has been confirmed in numerous studies (Paithankar et al., 2021). For instance, Cd can induce apoptosis in bronchial epithelial cells by activating the mitochondria-mediated intrinsic apoptotic pathway (Cao et al., 2021). Under higher concentrations of Cd exposure, kidney cells suffer damage, characterized by mitochondrial injury, cell swelling, and loss of basement membrane structure, while long-term low concentration exposure can lead to renal fibrosis (Thijssen et al., 2007). In the hypoxic environment associated with chronic obstructive pulmonary disease (COPD), Cd exacerbates damage to the kidneys and bones by overexpressing DMT-1, increasing the risk of COPD-related complications (Cirovic et al., 2022), while higher COPD incidence and mortality rates are associated with reduced HGS (Holden et al., 2021). Cd and Pb also disrupt the bioenergetics of human osteoblasts, reducing ATP production, mitochondrial complex activity, and aerobic respiration, and diminishing antioxidant defense enzyme activity and intracellular glutathione levels (Al-Ghafari et al., 2019). Exposure to Cd also induces oxidative stress, depletes cellular thiols, and stimulates apoptosis in mouse myoblasts (Zhang et al., 2019). Cd and Pb interfere with the normal function of calcium ions in cells, particularly affecting calcium-dependent proteins like TnC, disrupting intracellular metabolism and muscle fiber function, which may lead to metabolic disorders and toxic effects (Chao et al., 1990). Smoking-induced exposure to Cd and Pb can cause endothelial cells to release inflammatory and atherosclerosis-related cytokines, damaging endothelial cell processes and morphology, and

weakening their protective capabilities (van Strijp et al., 2023). Animal experiments show that the toxic effects of Pb on muscles include disrupting normal glucose metabolism, altering key enzyme activities and energy metabolism, potentially causing damage to muscle tissue structure, impacting muscle function and quality, and negatively affecting overall health (Das et al., 2020). Another animal study suggests that Pb exposure's damage to skeletal muscle function mainly includes reduced muscle mass, decreased exercise capacity, weakened muscle strength, disrupted lipid metabolism, increased inflammatory responses, and upregulated expression of pro-apoptotic genes, possibly due to lead's direct effects on muscle cell structure, metabolism, and inflammatory pathways (He et al., 2023).

A study focusing on residents near mining and smelting areas in Northwest China showed that the combined exposure to Cd and Pb is associated with systemic immune-inflammatory responses, which intensify with increasing levels of Cd and Pb co-exposure (Zhang et al., 2022). In a study on children, it was found that exposure to heavy metals such as Pb and Cd could disrupt the body's immune balance and induce inflammatory responses, adversely affecting health (Zhang et al., 2020). The toxic effects of Cd operate by disrupting normal cellular signaling pathways, particularly by activating the C/EBP-DDIT3 signaling pathway, inducing endoplasmic reticulum (ER) stress and inflammatory responses, damaging the normal functions of airway cells, and potentially leading to various pulmonary diseases such as chronic obstructive pulmonary disease (COPD) (Kim et al., 2017). Cd in tobacco smoke can activate inflammatory responses through free radical-mediated tissue damage (Milnerowicz et al., 2015). Another animal study indicated that the damage Cd causes to skeletal muscles mainly manifests as decreased muscle weight and strength, reduced exercise capacity, disrupted lipid metabolism, enhanced inflammatory responses, and increased expression of pro-apoptotic genes. These damages likely stem from Cd's direct toxic effect on muscle cells and the resulting inflammatory responses (He et al., 2023).

Pb is widely recognized as a potent neurotoxin, and a substantial body of research evidence suggests that exposure of the developing central nervous system (CNS) to Pb can lead to changes in cognition, motor skills, and behavior. Although its neurotoxic mechanisms are complex, Pb appears to alter the release of neurotransmitters, cause excitotoxicity, and ultimately lead to apoptosis (Stretesky and Lynch, 2004; Toscano and Guilarte, 2005). Epidemiological and clinical research data indicate that Cd can penetrate the blood-brain barrier, infiltrate the nervous system, and cause a range of severe neurological symptoms such as headaches, dizziness, olfactory disturbances, memory loss, polyneuropathy, attention deficits, and Parkinson-like symptoms (Viaene et al., 2000; Ciesielski et al., 2013). Exposure to Pb and Cd interferes with key processes in adult neurogenesis, inducing neurotoxicity by affecting adult neurogenesis and accelerating or exacerbating cognitive decline associated with neurodegenerative and/or aging processes (Wang and Matsushita, 2021). The intake of Cd and Pb affects the homeostasis and neuroprotective cascade reactions of astrocytes, including glutamate/GABA-glutamine transport, antioxidant mechanisms, and energy metabolism. Abnormalities in astrocyte pathways may promote or trigger neurodegenerative changes (Li et al., 2021). In an animal study, it was found that a mixture of Cd and Pb induced synergistic toxicity in astrocytes, potentially damaging the blood-brain barrier (BBB) and

leading to behavioral disorders in developing rats, such as hyperactivity, enhanced grip strength, and learning and memory deficits (Rai et al., 2010).

The common characteristic in the mechanisms by which Cd and Pb damage grip strength is their ability to induce oxidative stress in the body. This is accomplished by the production of excessive reactive oxygen species (ROS), leading to an imbalance in the cellular antioxidant regulatory system. This redox imbalance not only damages cellular structures and functions, including muscle cells, but can also directly affect grip strength. The cytotoxicity caused by Cd and lead, through various mechanisms such as mitochondria-mediated apoptosis and cell membrane damage, affects cell survival, which is crucial for maintaining the health of muscle cells as their functional state is directly linked to muscle strength. Studies suggest that Cd and lead, by affecting the structure and metabolic pathways of muscle cells, may reduce muscle function and quality, thereby weakening grip strength. Additionally, these metals can induce immune and inflammatory responses, which also adversely affect the health and functionality of muscle tissue. These findings highlight the importance of further research into the exposure to Cd and Pb and their impact on muscle health.

In terms of the differences in the mechanisms by which Cd and Pb damage grip strength, Pb is widely regarded as a potent neurotoxin, with particularly significant effects on the central nervous system. Pb exposure may alter the release of neurotransmitters and nerve conduction, which could indirectly affect the neural control of muscles, thereby impacting grip strength. In comparison, while Cd does affect the nervous system, its impact might not be as direct or pronounced as that of lead. Cd primarily affects the kidneys, liver, bones, and nervous system, and its damage may indirectly affect overall health status and muscle function through the impairment of these organs' functions. Pb is known for its significant damage to the nervous system, potentially causing direct effects on muscles by disrupting normal glucose metabolism processes and energy metabolism mechanisms. In contrast, the effects of Cd might be more related to the oxidative stress and cytotoxicity it induces. These differences reflect the complexity of the mechanisms of action of these metals within the body and suggest that future research needs to delve deeper into how these metals affect muscle function and overall health through different pathways.

The impact of Cd and Pb on HGS may involve a range of complex mechanisms, including oxidative stress, cytotoxicity, immune responses, and inflammation. Although both metals exhibit similarities in certain toxic mechanisms, they also show significant differences, particularly in their effects on the nervous system and specific metabolic pathways. Understanding these similarities and differences is crucial for the prevention and treatment of health issues caused by these heavy metals. Given these findings, the current focus should be on addressing the adverse effects of Cd and Pb on HGS and implementing appropriate preventive and intervention measures. This requires not only application in clinical medicine but also attention in public health policies and environmental management to reduce the exposure risk to these heavy metals and protect human health.

4.2.2 The protective mechanism of Se

Se, as a crucial trace element, plays an essential role in human health, especially in antioxidant activities and maintaining immune

functions. When exploring how Se can counteract the damage caused by Cd and Pb to HGS, several potential protective mechanisms can be considered.

Se plays a crucial role in enhancing the cell's antioxidant defense system and maintaining redox balance, which helps alleviate the damage caused by oxidative stress from Cd and Pb on muscle cells, thereby protecting HGS. Se, by improving antioxidant defense, immune function, and metabolic homeostasis, can reshape progressive and spontaneous physiological changes caused by oxidative stress, potentially preventing diseases and promoting healthy aging (Björklund et al., 2022). As a component of selenoproteins, the trace element Se performs an important antioxidant function in preventing muscle tissue damage (Pei et al., 2023). Additionally, Se enhances the activity of antioxidant enzymes, with its physiological effects primarily stemming from its role in selenoproteins (Avery and Hoffmann, 2018). Se-containing glutathione peroxidases help reduce damage caused by free radical reactions (Kieliszek and Błażej, 2016). Se-dependent glutathione peroxidases (GPX1-4 and GPX6) and thioredoxin reductases (TrxR1-3) can directly inhibit oxidative stress. Cytoplasmic GPX4 is crucial for embryonic development and cell survival, while GPX1 is the primary metabolic form in the human body to combat severe oxidative stress (Wu et al., 2012). GPX1 maintains redox balance by detoxifying reactive oxygen species (ROS) (Avery and Hoffmann, 2018). Studies suggest that ROS accumulated over a lifetime may lead to muscle loss, and selenoproteins like glutathione peroxidases can protect muscle cells from oxidative damage (Fernández-Lázaro et al., 2020).

Se has potential protective effects in preventing cell damage caused by heavy metals. *In vitro* studies show that the presence of Se in tissues can reduce the toxicity of Cd not only by storing and redistributing these toxic metals but also by forming Cd-Se complexes and binding with selenoprotein P, thereby decreasing the bioavailability of Cd (Siscar et al., 2014). Furthermore, Se may promote cellular repair and regeneration. By regulating intracellular metabolic processes, Se helps alleviate the toxic effects of heavy metals. It may assist in restoring metabolic imbalances caused by Cd and lead, maintaining the normal function of muscle cells, and thus preserving handgrip strength. For example, Se can regulate signaling pathways affecting cell survival to protect cardiac muscle cells (Shalihah et al., 2021). Se plays a crucial role in the regulation of thyroid hormones, controlling normal development, growth, and cell metabolism. These studies indicate that Se not only functions in antioxidant defense but also plays an important role in maintaining cellular structure and function, as well as promoting damage repair (Rosen and Liu, 2009).

Se may protect handgrip strength by alleviating inflammatory responses caused by Cd and Pb and promoting the repair processes post-inflammation. In the human body, Se primarily exists in the form of selenocysteine and selenoproteins, among which selenoproteins, acting as antioxidants, have significant anti-inflammatory effects. Particularly, one of the major selenoproteins, glutathione peroxidase (GPX), can effectively control the excessive production of free radicals at inflammation sites. Additionally, selenoprotein-S also plays an important role in regulating inflammatory cytokines (Hariharan and Dharmaraj, 2020). Through these mechanisms, Se helps to reduce the inflammatory response caused by heavy metals, alleviating damage to muscle tissue, thus aiding in maintaining or restoring normal muscle function and handgrip strength.

While research on Se's protective mechanisms against the neurotoxicity caused by heavy metals like Pb and Cd is not yet extensive, existing studies have shown that Se can significantly delay or mitigate certain neurotoxic effects of methylmercury, thus protecting motor and sensory functions (Heath et al., 2010). An animal study indicated that Se could alleviate the decline in glutathione levels and the reduction in acetylcholinesterase activity caused by arsenic. It also reduced oxidative stress and the production of reactive oxygen and nitrogen species, as well as lowered inflammation parameters. Overall, Se effectively mitigated behavioral disorders in rats caused by arsenic through its anti-inflammatory, antioxidant, and anti-apoptotic mechanisms (Adedara et al., 2020). The synergistic action of these mechanisms is crucial for protecting the nervous system from the adverse effects of heavy metals. Thus, the potential role of Se in neuroprotection deserves further investigation, especially its potential in mitigating neural damage caused by exposure to heavy metals.

In summary, Se exerts a protective effect by reducing the adverse impact of Cd and Pb on handgrip strength through its antioxidant properties, regulation of cellular metabolism, alleviation of inflammatory responses, and mitigation of neurotoxicity. This multifaceted protective role makes Se an important trace element in combating muscle damage caused by heavy metals. Consequently, judicious Se supplementation could enhance HGS. Nevertheless, an overabundance of Se may induce toxicity, potentially due to excessive Se triggering oxidative stress and generating harmful radicals that inflict cellular and organ damage. Se may also convert into deleterious selenite salts, perturbing cellular processes and producing toxic consequences. It is paramount to recognize that Se's antagonistic efficacy is contingent upon intake levels and individual variabilities. As such, when leveraging Se's antagonistic potential, its dosage and ensuing effects warrant meticulous consideration, and adjustments should be made attuned to specific circumstances. In essence, while Se offers a promising avenue to counteract the toxic effects of Cd and Pb, it should not be solely relied upon as a panacea. The impact of Cd and Pb on HGS is a complex process, involving multiple biological pathways. Se, as an important trace element, shows significant potential in countering the toxicity of these heavy metals. The findings of this research emphasize the importance of considering trace element interactions in environmental health studies and provide new perspectives for future research in this field. Future studies should further explore the interactions between Se and other trace elements, as well as how they collectively influence the impact of heavy metal exposure on human health.

4.3 Explanation for the subgroup analyses results

The subgroup analysis underscored the pronounced impact of gender and age on the association between HGS and blood metal mixtures (comprising Cd and Pb) across different Se quartiles. With respect to gender, females exhibited greater vulnerability to the negative implications of heavy metals on HGS. An increase in Se concentration notably mitigated this damage, and the protective effect began to diminish after reaching a certain threshold, demonstrating an initial enhancement followed by a decline. Conversely, males exhibited a nonlinear negative correlation, although it lacked statistical significance.

Such observations accentuate the differential gender-based effects of heavy metal exposure on HGS.

In terms of age, the elderly were notably more vulnerable to the adverse effects of heavy metals on HGS. With the increase in Se concentration, there is an initial strengthening followed by a subsequent weakening trend in Se's ability to mitigate the adverse effects of heavy metals on HGS in the elderly. For the middle-aged group, a discernible nonlinear negative trend was observed, but, akin to males, it did not attain statistical significance. This emphasizes the paramount role age assumes in the interplay between heavy metal exposure and HGS.

For populations at risk of diabetes, these findings highlight that the protective role of Se in mitigating the decline in HGS caused by heavy metal toxicity may be limited. This phenomenon suggests that in individuals with a risk of diabetes, the protective effect of Se against heavy metal-induced handgrip strength impairment might not be as significant as in those without a diabetes risk. Therefore, for people at risk of diabetes, it becomes even more crucial to avoid the impairment of grip strength caused by heavy metals. Furthermore, this indicates that future research should delve deeper into how the risk of diabetes influences the toxicity of heavy metals and the protective role of Se, as well as how to protect this specific population more effectively from the adverse effects of heavy metals.

The subgroup analysis of this study reveals the importance of considering factors such as gender, age, and diabetes risk when assessing the relationship between heavy metal (Cd and lead) exposure and HGS. This underscores the need to consider the variability in susceptibility and response to heavy metal exposure among different demographics and risk groups, offering a more nuanced understanding of the potential health impacts. These insights are invaluable for shaping future interventional approaches. The results illuminate the nuanced susceptibilities in HGS impairments, underscoring that, within specific concentration limits, Se effectively counteracts the negative effects of heavy metals on HGS. These insights serve as a foundational academic base to inform and refine targeted preventive and healthcare strategies.

4.4 Clinical implications

This study elucidates the pivotal relationship between Se and the adverse effects of Cd and Pb on HGS, offering substantial implications for clinical contexts. It underscores the imperative of risk assessment and management, particularly when individuals present with compromised grip strength in areas with heightened heavy metal levels. Such findings accentuate the importance of targeted interventions and the broader dissemination of public health advisories. Considering the heightened susceptibility of females, elderly individuals, and diabetes patients to the toxicity of heavy metals, special attention should be given to these populations. When formulating proactive measures, risk variations associated with gender, age, and diabetes patients should be considered. Moreover, policy recommendations could push for stringent controls on heavy metal exposure, especially in settings predominantly occupied by these vulnerable groups. A deeper dive into research is requisite to delineate the causal dynamics among Se, Cd, and Pb with HGS, and to unpack their intertwined biological mechanisms.

In essence, Se stands as a potent counteragent to the toxicity induced by heavy metals such as Cd and Pb. This provides a robust academic foundation to mitigate HGS afflictions and amplify preventive awareness. It is pivotal to understand that while Se can play a therapeutic role in neutralizing heavy metal toxicity, its intake should be judicious. A balanced Se intake can be adjunctive in treatment, emphasizing the salience of routinely monitoring heavy metal levels in the body. This facilitates early identification of potential toxic threats. However, any intervention should be judiciously overseen by healthcare professionals to ensure Se consumption remains within safe limits.

4.5 Limitations and strengths

The study's foremost strength stems from its application of multiple models to probe the effects of both isolated and combined exposures to Se, Cd, and Pb with HGS, thereby amplifying the research's breadth and depth. Bolstering the study's credibility, it leveraged the nationally representative NHANES dataset, benefitted from a substantial sample size, and meticulously adjusted for numerous potential confounders, which consolidates the trustworthiness of its conclusions.

However, the research is not devoid of limitations. The study's cross-sectional design inhibits discerning a definitive causal link between Se, Cd, and Pb with HGS. Even with rigorous accounting for known confounders, the potential influence of unquantified or overlooked confounding factors cannot be dismissed. The assessment of HGS, while insightful, has inherent limitations such as localization, singular indices, individual variations, testing inaccuracies, physiological shifts, and extrinsic influences, potentially introducing biases. Another point of contention is the generalizability of the findings. Since the data primarily pertains to American adults, extrapolating these conclusions to global populations is circumspect. Although several studies have ventured into the relationships between Se, Cd, or Pb and functions like lung capacity, comprehensive human studies that integrate all these elements remain elusive. The intricate interplay and mechanisms of Se's interaction with bloodborne heavy metals, such as Cd and Pb, are yet to be demystified. As a forward-looking recommendation, the validation of this study's conclusions would benefit immensely from longitudinal cohort studies boasting expansive and diverse sample sizes. The absence of forearm circumference data is a limitation in our study, as it could have a direct biological correlation with HGS. Therefore, not including this variable might limit our comprehensive understanding of factors influencing HGS. Secondly, iron, a key element for hemoglobin synthesis, is crucial for maintaining normal muscle function and strength. Both iron deficiency and excess can adversely affect muscle health, thereby indirectly impacting handgrip strength. However, our study did not include data on iron levels, suggesting that future research should consider iron levels to understand the impact of different nutrients and environmental factors more fully on handgrip strength. Lastly, the combined effects of Se with other trace elements, such as zinc and copper, which may have similar or superior biological effects, might be more effective in improving HGS. This indicates that future research should more comprehensively consider and investigate the interactions and synergistic effects of various trace elements to gain a deeper understanding of how these elements collectively influence muscle function and handgrip strength.

5 Conclusion

This study primarily investigated the effects of Se, Cd, and Pb on HGS. The results showed that, after adjusting for confounding factors, Se was positively correlated with HGS, while Cd and Pb were negatively correlated. Further analysis revealed that higher levels of Se could mitigate the negative impact of Cd and Pb on HGS. Additionally, there was an inverted U-shaped relationship between Se and HGS, indicating that the benefits of Se on HGS tend to plateau beyond approximately 200 µg/L. Stratified analysis revealed that as Se levels increased, the negative effects of Cd and Pb on HGS gradually diminished. However, in populations at high risk of diabetes, the role of Se in counteracting the HGS decline induced by heavy metals was limited, suggesting that more detailed management of heavy metal exposure is needed in this group. The study underscores the important role of Se in alleviating the adverse effects of Cd and Pb on HGS, particularly its potential protective effects in specific high-risk groups. This provides a new perspective for future research to explore optimal strategies for Se supplementation and its synergistic effects with other nutritional and environmental factors.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: <https://www.cdc.gov/nchs/nhanes/index.htm>.

Author contributions

YL: Investigation, Software, Writing—original draft, Writing—review and editing. JW: Investigation, Software, Writing—original draft, Writing—review and editing. TW: Investigation, Software, Writing—original draft, Writing—review and editing. HL: Formal Analysis, Writing—original draft. CY: Formal Analysis, Writing—original draft. JL: Formal Analysis, Writing—original draft. YW: Formal Analysis, Writing—original draft. JF: Formal Analysis, Writing—original draft. SF: Conceptualization, Writing—original draft, Writing—review and editing. SZ: Conceptualization, Writing—original draft, Writing—review and editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the program of China Scholarships Council (No. 202006550005).

Acknowledgments

Data of the National Health and Nutrition Examination Survey (NHANES) was used in this study. It is with great appreciation that we thank the NHANES research team, the field team, and every respondent who took part in this study.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2023.1324583/full#supplementary-material>

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OPEN ACCESS

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RECEIVED 23 January 2024

ACCEPTED 04 March 2024

PUBLISHED 19 March 2024

CITATION

Afzal A and Mahreen N (2024), Emerging
insights into the impacts of heavy metals
exposure on health, reproductive and
productive performance of livestock.
Front. Pharmacol. 15:1375137.
doi: 10.3389/fphar.2024.1375137

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Emerging insights into the impacts of heavy metals exposure on health, reproductive and productive performance of livestock

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Heavy metals, common environmental pollutants with widespread distribution hazards and several health problems linked to them are distinguished from other toxic compounds by their bioaccumulation in living organisms. They pollute the food chain and threaten the health of animals. Biologically, heavy metals exhibit both beneficial and harmful effects. Certain essential heavy metals such as Co, Mn, Se, Zn, and Mg play crucial roles in vital physiological processes in trace amounts, while others like As, Pb, Hg, Cd, and Cu are widely recognized for their toxic properties. Regardless of their physiological functions, an excess intake of all heavy metals beyond the tolerance limit can lead to toxicity. Animals face exposure to heavy metals through contaminated feed and water, primarily as a result of anthropogenic environmental pollution. After ingestion heavy metals persist in the body for an extended duration and the nature of exposure dictates whether they induce acute or chronic, clinical or subclinical, or subtle toxicities. The toxic effects of metals lead to disruption of cellular homeostasis through the generation of free radicals that develop oxidative stress. In cases of acute heavy metal poisoning, characteristic clinical symptoms may arise, potentially culminating in the death of animals with corresponding necropsy findings. Chronic toxicities manifest as a decline in overall body condition scoring and a decrease in the production potential of animals. Elevated heavy metal levels in consumable animal products raise public health concerns. Timely diagnosis, targeted antidotes, and management strategies can significantly mitigate heavy metal impact on livestock health, productivity, and reproductive performance.

KEYWORDS

heavy metals, toxicity, health, production performance, livestock

Introduction

Technological developments have raised serious concerns about environmental safety as uncontrolled industrialization without emission control has put livestock health at risk (Prata et al., 2021). The livestock sector, an indispensable part of the global economy, is facing challenges because the production potential of animals depends on their health status, influenced by infectious and non-infectious diseases (Bailey et al., 2021). Heavy metal

poisoning is one of the major causes of non-infectious diseases that adversely affect animal health. The risk of vulnerability is increasing due to widespread industrial, agricultural, domestic, technological, and medical applications of heavy metals (Vardhan et al., 2019).

Heavy metals like Zn and Cu are incorporated in animal feed as they play pivotal roles in immune function, growth, and metabolism (Hill and Shannon, 2019). These metals also serve as cofactors for numerous enzymes involved in protein synthesis and energy metabolism, thus promoting efficient nutrient utilization and growth in animals (Duffy et al., 2023). Moreover, Fe and Mn are indispensable for the formation of hemoglobin and enzymes involved in oxygen transport and antioxidant defense mechanisms (Pajarillo et al., 2021; Swecker, 2023). Adequate levels of these metals in livestock diets ensure optimal oxygen supply to tissues, which is particularly crucial for high-producing animals like dairy cows.

The escalating human activities contribute to a drastic increase in heavy metal addition to the environment, posing a continuous threat to livestock through water and soil pollution (Maurya et al., 2019; Ghazzal et al., 2022). The persistent exposure to these contaminants results in adverse health effects in livestock (Boudebbouz et al., 2023; Akbar et al., 2024). Additionally, the transfer of heavy metals from animals to humans through the food chain raises concerns about food safety and public health (Aftab et al., 2023; Noor et al., 2024).

The issue of metal toxicity has become a growing concern from evolutionary, ecological, environmental, and nutritional aspects particularly in the less developed countries of Africa, South Asia, and parts of Latin America (Pandey et al., 2014; Diarra and Prasad, 2021; Samy et al., 2022). In these countries, arid and semi-arid regions face particular challenges with metal pollution due to factors such as limited water resources, which concentrate pollutants, and the potential for windblown dust to spread contaminants over large areas (Chowdhury et al., 2016; Samy et al., 2022). Some countries in transition or with rapidly growing industrial sectors also face the challenge of heavy metal pollution due to lack of environmental regulations and limited resources for pollution control (Ding, 2019; Dutta et al., 2022; Patwa et al., 2022). While highly developed countries like the United States, China, Russia, and certain European nations have experienced issues with heavy metal contamination in soil, water, and air due to historical industrial practices (Li et al., 2014; Zhou et al., 2020). Altitude itself is not a direct factor in heavy metal pollution. However, certain activities more common at higher altitudes, such as mining in mountainous regions, can lead to localized heavy metal contamination. Additionally, factors like precipitation patterns and soil characteristics influenced by altitude can affect the transport and fate of metals in the environment (Giri et al., 2020).

The pervasive and mutinous nature of metal contaminants poses a potential risk for different animal populations. The distinct symptoms of metal poisoning in livestock include central nervous system (CNS) disorders, liver and kidney problems, reproductive failure, endocrine abnormalities, depression, and vision disturbances (Massányi et al., 2020; Rehman et al., 2021). If not diagnosed and treated properly, heavy metal exposure leads to complex problems with high morbidity and mortality rates. The sudden exposure to metals

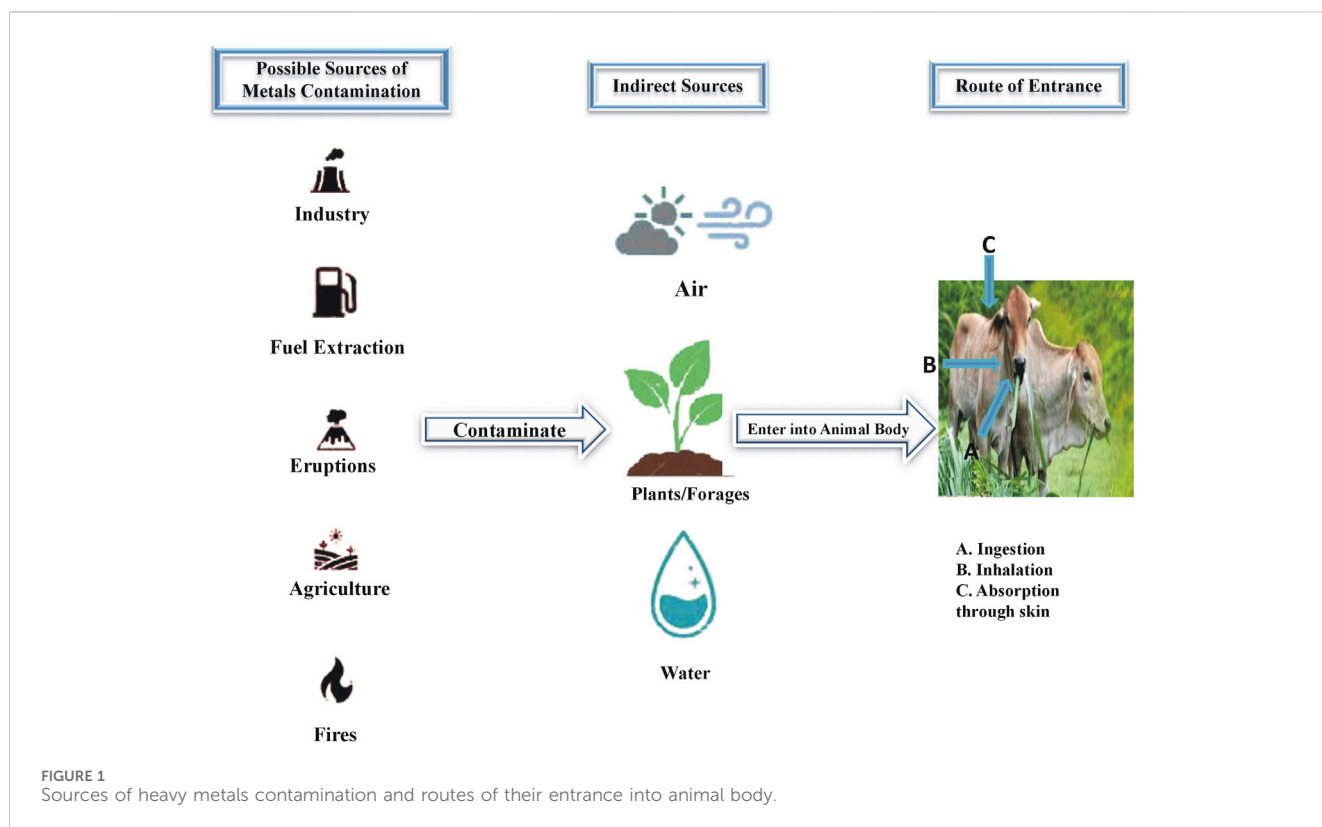
cause acute toxicity, with the severity of health issues depending on individual susceptibility, exposure route and duration, and the type and form of the element (Khan et al., 2016; Sinha et al., 2022). The pathophysiology of metals involves oxidative stress characterized by the generation of Reactive Oxygen and Reactive Nitrogen Species (ROS and RNS), reduction in intracellular free-radical scavengers and antioxidant stores, and decrease in enzyme capacity for detoxification of ROS. This leads to oxidative stress causing immunosuppression and a reduction in body condition scoring of domestic animals (Paithankar et al., 2021; Sun et al., 2022). Livestock populations are negatively influenced by exposure to excessive levels of harmful metals (e.g., Pb, Cd) or inadequate amounts of essential trace elements (e.g., Se and Mo) (Rajendran, 2013).

Ionic forms of metals exhibit high reactivity, engaging with biological systems in diverse ways that result in significant toxicological implications (Brandelli, 2020; Patel et al., 2021). The primary livestock species affected by metal poisoning in this scenario is cattle, mainly nourished with indigenously cultivated feed (Wang et al., 2013; Sharifian et al., 2023). To evaluate the potential effects of pollutants on livestock and measure contaminant intake in humans through milk and meat, it is essential to understand the concentrations of harmful metals in feed (Tortajada and Zhang, 2021; Ullah et al., 2023).

Research on the impacts of heavy metals exposure on the health, reproductive, and productive performance of livestock has garnered significant attention in recent years. Several published reviews have explored the diverse effects of heavy metals such as Pb, Cd, Hg, and As on various aspects of livestock physiology and productivity (Martinez et al., 2020; Sun et al., 2022; Duffy et al., 2023; Sadeghian et al., 2024). These reviews have elucidated the mechanisms by which heavy metals enter the animal body, their accumulation in tissues, and the resultant adverse effects on health. Some studies have highlighted the detrimental effects of heavy metals on immune function, oxidative stress, and reproductive functions in different livestock species (Garcia et al., 2017; Wang et al., 2019; Patel et al., 2021; Tahir and Alkheraije, 2023). Heavy metal contamination in livestock environments poses not only health risks to animals but also potential hazards to human consumers through the food chain. As such, this topic holds immense importance for informed decision-making in agricultural and veterinary practices. The study will comprehensively provide emerging insights and recent research findings regarding the effects of heavy metals on livestock. By collating and analyzing the latest scientific literature, this review aims to provide an overview of the current understanding of heavy metal toxicity in livestock and its implications for animal health and productive performance. Additionally, the review will explore potential mitigation strategies and areas for future research, contributing to the ongoing discourse on sustainable livestock management in the face of environmental challenges.

Heavy metals and their biological significance

Metals are classified broadly into highly toxic, less toxic, essential, and non-essential categories. Essential metals like Fe,



Zn, Mn, and Co are required in trace amounts for regulating various physiological functions such as hormone synthesis, oxygen and electron transportation, fertility, antioxidant defense, and immunity in livestock animals (Li et al., 2021). However, their presence beyond a certain limit in the biological system potentiates toxicity.

Highly toxic metals like As, Cu, Hg, Cd, and Pb can exert adverse effects even in minute concentrations without providing any beneficial biological impacts in animals (Li et al., 2014). Toxic metals impair the primary cellular functions by imitating the role of essential metals and this leads to their bioaccumulation. The elemental nature of metals affects the process of their biotransformation and detoxification through metabolic pathways that result in the breakdown of toxic atomic species into a less toxic or non-toxic species. In addition, the non-biodegradable nature of these metals further exacerbates the condition of toxicity (Florea et al., 2005).

Sources of heavy metals contamination

Sources of heavy metals contamination vary widely for livestock animals (Figure 1), with industrial effluents emerging as a significant contributor (Elheddad et al., 2021). Wastewater from industries introduces hazardous pollutants like Cr into the soil and water bodies (Dotaniya et al., 2014). This contamination poses threats to livestock as they may drink poisonous water or graze on polluted pastures. Similarly, automobile emissions contribute to metal contamination

along roadsides. Livestock grazing near roads exposed them to metal pollutants that adversely affect their health (Ghorani-Azam et al., 2016).

Moreover, the application of fertilizers including phosphate fertilizers and organic materials (sewage sludge and animal manure) introduces heavy metals into agricultural soils (Rizzardini and Goi, 2014). Livestock may consume crops grown in such contaminated soils, resulting in the bioaccumulation of heavy metals in their tissues. Additionally, mining activities release toxic metals (As, Hg, and Pb) into the environment (Okerefor et al., 2020). Animals in mining areas may face direct exposure to these pollutants through soil, water, and vegetation, impacting their health, productivity, and welfare.

Toxic metal contents in livestock feedstuffs

Livestock exposure to toxic metals is significantly influenced by husbandry practices. Sometime different dietary supplements and ingestion of contaminated soil also contribute to potential exposure to heavy metals. While evaluating the concentration of heavy metals in primary feed ingredients, it is crucial to consider husbandry practices as contributing factors to metal exposure. This consideration is essential to limit the toxic effects on animal health and the potential transfer of these contaminants to human feed. Providing a comprehensive review of toxic metal contents in animal feed proves challenging due to restricted information regarding sample description, analysis methods, and data expression. The limit of toxic heavy metals in both individual

TABLE 1 Amount (mg/kg DM) of heavy metals in animal feed components.

Feed components	Heavy metals			
	Cadmium	Lead	Arsenic	Mercury
Barley Grain	0.11	0.97	0.01	0.006
Citrus Pulp Meal	0.19	0.76	0.04	0.002
Fish Meal	0.4	0.52	4.7	0.1
Maize Grain	0.06	0.56	0.26	0.026
Rapeseed	0.15	0.6	0.08	0.031
Soya Bean Meal	0.07	0.93	0.022	0.055
Sugar Beet Pulp	0.14	1.47	0.36	0.028
Sun Flower Meal	0.41	0.37	0.001	0.003
Mineral Supplements	0.58	3.38	6.8	0.02
Wheat	0.19	0.26	0.043	0.003
Meat and Bone Meal	0.063	0.81	0.005	0.15
Fish Oil	0.021	0.14	7.6	0.03
Oil Seed Meals	0.005	0.06	0.09	0.007
Cereal and By Products	0.03	0.048	0.06	0.011
Amount (mg/kg DM) of Heavy Metals in Animal Forages				
Forages	Heavy Metals			
	Cadmium	Lead	Arsenic	Mercury
Grass/Herbage	0.62	4.93	0.12	0.071
Hey	0.73	3.89	0.05	0.18
Grass Silage	0.09	2.02	0.12	0.023
Maize Silage	0.28	2.19	0.05	0.007
Different Straws	0.15	0.033	0.05	0.026
Alfalfa	0.057	0.21	0.38	0.005
Amount (mg/kg DM) of Heavy Metals in Complete Animal Feeds				
Animals	Heavy Metals			
	Cadmium	Lead	Arsenic	Mercury
Ruminants	0.11	0.34	0.27	0.012
Poultry	0.16	0.87	1.83	0.039
Pigs	0.09	1.03	0.62	0.032
Horses	0.21	0.13	0.39	0.022
Mink	0.003	0.28	0.061	0.053
Rabbits	0.14	0.29	0.09	0.031
Rodents	0.36	0.002	0.73	0.05
Dogs and cats	0.13	0.04	0.002	0.02

feed ingredients and commercially manufactured compound feeds is presented in Table 1 based on European Union (EU) organization standards (Adamse et al., 2017). Limited literature

outside the EU provides information on toxic metal residues in livestock feedstuffs. Such data is often derived from samples collected at a very local level.

Livestock husbandry practices related to toxic metal exposure

Livestock husbandry practices, particularly those involving feed supplements and their waste management, contribute to elevated levels of toxic metal exposure in animals, posing risks to their health and production (Wang et al., 2013). Feed supplementation is a primary pathway for toxic metal introduction in animal production systems. Heavy metals such as Cu and Zn are often added to animal feed to promote growth and boost immune function (Harvey et al., 2021). These metals are essential in trace amounts, over-supplementation or improper formulation of feed can lead to accumulation in animal tissues, potentially reaching levels harmful for the animals themselves and as well as consumers of their products.

Furthermore, the disposal of livestock waste presents another avenue for toxic metal contamination. Manure from animals fed supplemented diets can contain elevated levels of heavy metals, which may leach into soil and water systems if not managed properly. As a result, crops grown in these environments can uptake these metals, perpetuating the cycle of contamination (Liu et al., 2020a). In addition, the spreading of manure on agricultural fields as fertilizer can introduce heavy metals into the food chain, posing risks to human health through consumption of contaminated crops or water sources (Qin et al., 2021). The exposure of animals to toxic heavy metals during husbandry practices also depends on the type of production system (extensive or intensive) under which they are housed. Therefore, effective waste management strategies are crucial to mitigate the spread of toxic metals from livestock operations to the broader environment.

Extensive production system

Regions of the world where animals are kept under extensive production systems, such as parts of South America, Africa, and Asia, are particularly vulnerable to heavy metal contamination due to factors such as industrial activities, mining operations, and improper waste disposal practices (Modernel et al., 2019; Otte et al., 2019; McAllister et al., 2020). The impact of heavy metals on livestock health and performance in these regions is most pronounced during the dry or winter seasons when forage availability is limited, and animals are forced to graze on contaminated vegetation or drink from contaminated water sources. During these periods, livestock animals experience heightened levels of heavy metal exposure, exacerbating the negative effects on their efficiency in extensive production system (Adegoke and Abioye, 2016; Johnsen and Aaneby, 2019). Prolonged exposure to elevated levels of these metals has led to various health issues, including gastrointestinal disturbances, liver and kidney damage, and neurological disorders. The presence immune system of animals is also compromised, reducing their resistance to pathogens and making them susceptible to infectious diseases (Villalba et al., 2016; Chen et al., 2022).

The heavy metal poisoning also disrupts the productive performance of animals in extensive system due to poor nutrient absorption and utilization. This leads to reduced feed efficiency, slower growth rate and decreased milk and meat production

(Miroshnikov et al., 2021). Moreover, metal toxicity also causes metabolic disorders that further compromise the profitability of extensive livestock operations. In severe cases, metal poisoning results in acute illness and death, posing significant welfare concerns for animals (Okerefor et al., 2020). Effective management strategies, including regular monitoring of soil and water quality, implementation of proper waste management practices, and targeted nutritional interventions, are essential to mitigate the risks associated with heavy metal contamination and ensure the sustainability of extensive livestock farming operations (Qin et al., 2021).

Intensive production system

Non-ruminant (pigs and poultry) animal species whose diet entirely consists of concentrates and ruminant animals (feedlots and milk production cattle) with a high rate (90%) of concentrates in their feed are managed under intensive production system (Moorby and Fraser, 2021). The feed for animals in this system is composed of various materials selected from national and international markets based on cost, availability, and nutritional suitability according to the physiological requirements of the animals.

The complete feed given to these animals is supplemented with specific feed additives and minerals that contain higher toxic metal contents compared to their basic feed. Due to low inclusion rate, the contribution of these supplements to total metal intake (TMI) is minimal, except for certain elements like Hg and As in marine-originated feedstuffs (Elliott et al., 2017).

In the intensive production system, toxic metal exposure is easily calculable by multiplying the toxic metal contents in the complete feed by the amount ingested. Furthermore, the diets in this system are highly standardized resulting in lower intra- and inter-farm variability of toxic metal exposure through the diet compared to the extensive system (Wang et al., 2013).

Health risks and toxic effects of heavy metals in animals

Health risks and toxic effects of heavy metals in animals depend on the type and form of the metal, the extent of exposure, sex, age, nutritional and physiological status of the exposed animal, and the route of poisoning. Due to their non-biodegradable nature heavy metals tend to accumulate in vital organs more rapidly than they are metabolized and excreted. This bioaccumulation of heavy metals poses a significant health risk adversely affecting the production performance of domestic animals. The potential health menaces caused by various heavy metals are described below.

Arsenic

Arsenic (As) known as the “Poison of Kings and the King of Poisons,” is an ancient toxin. Domestic animals face As exposure from contaminated pastures, dipping and spraying chemicals, pesticides, herbicides, growth promoters, and feed additives. Groundwater and metal-bearing ores are natural sources of As

(Constable et al., 2017). After absorption As accumulates in the liver then slowly dispenses to the tissues of other organs like kidneys, spleen, and lungs. Animals in As toxicity exhibit gastrointestinal, nervous, and cardiovascular symptoms. The acute poisoning cases show restlessness, abdominal pain, respiratory distress, and vomiting (Gupta et al., 2021).

A meta-analysis of 156 cases in 16 outbreaks revealed common clinical signs in cattle including ataxia, diarrhea, dehydration, respiratory stress, and death (Faires, 2004). Hematuria, azotemia, increased hematocrit and liver enzyme activities were observed as biochemical changes (Bertin et al., 2013). Survival in acute As toxicity is possible with aggressive fluid therapy and antidote administration. Chronic As toxicity manifests as capricious appetite, weight loss, mucosal lesions, mouth ulceration, and reduced milk yield in dairy cattle. Biochemical changes in subclinical cases include high blood as levels, low plasma proteins and erythrocyte count (Rana et al., 2010).

Quantification of As in stomach contents and tissues is a standard test for diagnosis. While for sample collection liver is regarded as the best organ for acute poisoning and kidneys for sub-acute or chronic cases (Constable et al., 2017). Levels exceeding 3 ppm in liver and kidneys indicate toxicity. As also appears in urine samples a few days after exposure (Sharaf et al., 2020). The diagnostic range for As in feces is 10–20 mg/g, while As quantification in hair and urine serves as a biomarker for monitoring chronic exposure in livestock (Das et al., 2021; Tiffarent et al., 2022).

Lead

Lead (Pb) recognized as the first toxic element that poses a higher frequency of poisoning in animals than any other metal (Gupta, 2019). This issue spans worldwide affecting all species of livestock, with cattle and calves being susceptible while caprine and swine show tolerance. Cattle due to their indiscriminate eating and licking habits ingest Pb-bearing objects from their environment including agricultural, industrial, and domestic wastes resulting in acute Pb poisoning (Liu et al., 2020b). Apart from natural exposure anthropogenic activities also introduce Pb into the food chain through contaminated feed and water, burning of fossil fuels, paint industries, and combustion of coal. (Zyambo et al., 2022). Pastures near highways may carry up to 390 ppm Pb (Anil et al., 2021). Organic Pb compounds are more toxic than inorganic forms and animals with essential dietary mineral deficiencies are more prone to lead toxicity (Prabhakar et al., 2012; Su et al., 2022).

Pb has ability to cross blood-brain and placental barriers, so it adversely affects the brain and fetus of exposed animals. Clinical signs manifest due to chronic exposure when Pb levels surpass saturation limits in natural sinks like bones (Constable et al., 2017). Pb affects various physiological functions leading to gastrointestinal damage, neurotoxicity, liver and kidney dysfunctions, oxidative stress, and disruptions in enzyme systems (Finnie et al., 2011; Patra et al., 2011; Slivinska et al., 2020). Clinical signs in Pb poisoning mainly result from toxic effects on the CNS, gastrointestinal tract (GIT), and haemopoietic system. Neurological signs include bellowing, blindness, dullness, head pressing, opisthotonus, convulsions, and coma. Gastrointestinal

signs include distension, pain, cramping, and diarrhea. Sub-acute Pb toxicity presents anorexia, dullness, depression, weight loss, eye sight loss, staggering, in co-ordinations, and sometimes circling, followed by death (Krametter-Froetscher et al., 2007; Mukherjee et al., 2022). Pb also affects the reproductive system impacting animal productivity (Okerefor et al., 2020). Pb exposure alters essential trace mineral profiles, with cows exhibiting lowered blood Cu and Fe at blood Pb levels >0.60 µg/mL (Patra et al., 2006). Some studies show higher tolerance to Pb in sheep and goats than cattle as these animals excrete higher concentration of Pb in their excretions (Makridis et al., 2012). Goats being highly resistant may show signs of CNS involvement following long-term exposure (Swarup et al., 2005).

Diagnosis is based on significantly high Pb concentrations in blood, liver, and kidney samples (Oluokun et al., 2007). Management and prevention involve identifying sources and adopting preventive measures providing supportive care with chelation therapy and ensuring food safety. Calcium versenate is an effective antidote with combination therapy showing promising results. Recovery may occur rapidly in less severe cases but Pb concentrations in tissues remain high posing a potential source of residues in the food chain (Siddiqui and Rajurkar, 2008).

Mercury

Mercury (Hg) exists naturally in elemental, organic, and inorganic forms in the environment. Historically, it was used in traditional Chinese and Indian medicines. Different sources of Hg including vinyl chloride, chlor-alkali, electrical and electronic batteries, posing a potential threat to marine ecosystems through industrial effluents (Barregard et al., 2022). Domestic animals are less prone to Hg poisoning. However, accidental cases may occur especially in cattle (de Los Santos et al., 2023). Hg exposure stems from ingestion of organic or inorganic compounds, while toxicity observed at an average daily intake of 10 mg per kg body weight. Sheep and horses also exhibit toxic effects under certain intake levels (Constable et al., 2017). Hg absorption occurs through the GIT and then it affects other vital organs of the body (Bubber, 2019). Hg is slowly excreted through feces, milk, and urine, making the liver, kidneys, and milk from poisoned animals unsafe for human consumption (Bubber, 2019; de Los Santos et al., 2023).

Some organic Hg compounds are neurotoxic, affecting the nervous system even at lower concentrations (Ceccatelli et al., 2010). Inorganic Hg adversely affects the GIT mucosa causing vomiting, diarrhea, and colic, with acute poisoning signs in cattle including sudden behavioral changes, blindness, convulsions, and death (Krametter-Froetscher et al., 2007). Diagnostic importance lies in Hg concentration in blood, urine, brain, kidney, liver, and hair. Kidneys are ideal for detecting Hg poisoning in deceased animals, with a tissue concentration of >5 ppm regarded as clinically toxic. Blood Hg concentration provides more information about acute exposure, while urine samples determine inorganic Hg poisoning. Combining blood, urine, and hair samples provides accurate information about different forms of Hg exposure (Gupta, 2018). Effective treatment involves using 2,3-dimercaptopropanesulphonate (DMPS) and Meso-2,3-dimercaptosuccinic acid (DMSA) which mobilize Hg deposits for excretion in urine. Se

supplementation in the diet proves useful in protecting against organic and inorganic Hg toxicity in poultry, as Se and Hg are mutual antagonists. Phytoremediation, utilizing plant species like *Brassica juncea* has emerged as a proven approach to reduce Hg contamination in soil (Ekino et al., 2007). However, multidisciplinary research is essential to manage Hg toxicity and understand various treatment mechanisms.

Cadmium

Cadmium (Cd) a ductile and soft element with toxic properties, poses severe damage to vital organ systems (Rahimzadeh et al., 2017). It enters the environment through natural (volcanic activities, mineral ores, forest fires) and anthropogenic (processing facilities, non-ferrous metal smelters) sources, often released in combination with metals like Pb and Zn (Robards and Worsfold, 1991; Suhani et al., 2021). It is usually found as an impurity along with Zn and Pb deposits and therefore can be extracted as a byproduct during the smelting process of these metals. Rock phosphate fertilizer and sewage sludge leakage contribute to soil contamination leading to Cd transfer to plants and the food chain (Rahimzadeh et al., 2017). Domestic animals are exposed to Cd mainly through ingestion of plants grown in contaminated soils or in the vicinity of industrial units emitting Cd in the environment. Cd is mainly accumulated in leaves that act as a potential source of exposure to grazing animals (Madejon et al., 2009). Studies in Croatia and China revealed Cd concentrations exceeding limits in kidneys and liver, indicating environmental contamination (Cai et al., 2009; Bilandzic et al., 2010). Water from deep wells in certain areas may also carry high Cd levels (Perez-Carrera et al., 2016).

Cd ingestion causes acute liver damage and nephrotoxicity, while chronic exposure leads to organ accumulation and various clinical signs including in-appetence, weight loss, hoof and hair abnormalities (Constable et al., 2017). Severe vascular degeneration and necrotic changes occur in vital organs around industrial areas (Gumasta et al., 2018). Experimental administration in sheep resulted in nephropathy, anemia, bone demineralization, congenital defects, stillbirths, and abortion (Constable et al., 2017).

Limited exposure to Cd leads to subclinical effects like immunotoxicity, oxidative stress, reduced reproductive performance, endocrine disruption, altered micronutrient profiles, and poor weight gain (Swarup and Dwivedi, 2002). Long-term exposure causes lipid peroxidation, inhibits antioxidant enzymes, indicating oxidative damage in liver, kidneys, and testes (Wang et al., 2020). Ca, Fe, and Zn deficiency increases susceptibility to Cd toxicity, while Se supplementation protects the liver and kidneys (Alonso et al., 2004; Jemai et al., 2020). Including Mo and Fe in the diet prevents Cd poisoning signs (Smith and White, 1997; Alonso et al., 2004; Young et al., 2019).

Copper

Copper (Cu) is an essential element crucial for various enzyme systems in the body, but excessive intake can lead to severe toxicity in farm animals particularly in ruminants lacking efficient regulatory mechanisms for Cu (Trouillard et al., 2021). Sheep are

highly susceptible to Cu poisoning while cattle show relative resistance, though cases are increasing especially in dairy cattle (Lopez-Alonso et al., 2017).

Ruminants have poor homeostatic mechanisms for Cu, storing excess in the liver. When exposed to levels beyond physiological requirements they fail to regulate it leading to severe Cu toxicity (National Research Council, 2005; Almeida et al., 2022). Acute poisoning in sheep and young calves occurs with an intake of 20–100 mg/kg of body weight (BW), while adult cattle require 200–800 mg/kg BW. Chronic poisoning in sheep results from a daily intake of 3.5 mg/kg BW from pastures with 15–20 ppm Cu on a dry matter basis (Gupta, 2018). Goats tolerate higher Cu dietary intake (640 mg/kg BW) and toxic doses for non-ruminants are higher than this (National Research Council, 2005; Constable et al., 2017). Cu absorption varies in animals influenced by diet, husbandry practices, and breed types (Lopez-Alonso and Miranda, 2020).

Acute Cu poisoning in animals exhibits signs like gastric pain, diarrhea, anorexia, excessive salivation, dehydration, collapse, and incoordination, with some survivors developing icterus and dysentery (Elliott et al., 2020). Chronic poisoning manifests as hemolytic conditions, causing thirst, anorexia, depression and jaundice along with nervous signs (Quevedo et al., 2022). Cattle and buffaloes in chronic Cu poisoning show apathy, hemoglobinuria, reduced ruminal movements, dehydration, mild diarrhea, and icteric mucus membrane. Cu toxicity affects reproductive performance causing disorders related to pregnancy and influencing estrogen receptors (Hinde, 2021).

Diagnosis involves assessing Cu concentrations in tissues with liver biopsy being the most authenticated method. Serum Cu and cerulo-plasmin levels in live animals are commonly used parameters. Toxic levels in liver and kidneys are >250 ppm and 10 ppm in cattle, and >250 ppm and >18 ppm in sheep (Lopez-Alonso et al., 2006; Dalefield, 2017). Treatment involves using Mo and chelating agents (Antonelli et al., 2016; Mecitoglu et al., 2017; Martins et al., 2020). Zn supplementation is effective in preventing excessive Cu accumulation in sheep (Minervino et al., 2009). The potential health risks of heavy metals along with their maximum dose limits are summarized in Table 2.

Effects of heavy metals on reproductive performance

Male reproductive performance

Heavy metal toxicity has adverse effects on male reproductive performance impacting sperm maturation, concentration, and motility as shown in Figure 2 (Ilieva et al., 2020). In some livestock animal species sperm antioxidant defense system is poorly developed, so heavy metals disrupt normal motility and symmetry by accessing flagellum proteins through the sperm plasma-lemma (Kanous et al., 1993). Metal exposure in other species negatively affects the expression of metallo-thionein mRNA in testicular tissue crucial for protecting spermatogenesis from adverse effects of harmful substances (Sheng et al., 2015).

Mammalian testes are particularly susceptible to metal toxicities undergo remodeling of gene expression, alkalization of luminal fluid, and destabilization of sperm chromatin (Machado-Neves,

TABLE 2 Heavy metals and their associated health risks in animals.

Heavy metal	Form	Sources	Entry routes	Maximum daily dose ($\mu\text{g/day}$) limits		Associated symptoms	Pronounced health effects	References
				Parenteral	Oral/ Topical/ Mucosal			
Arsenic (As)	Inorganic	Pesticides, Contaminated water	Ingestion, Inhalation	1.5	15	Skin lesions, Gastrointestinal issues, Neurological disorders	Skin cancer, Diabetes Cardiovascular disease	Faires, (2004); Rana et al., (2010); Bertin et al., (2013); Gupta et al., (2021)
Lead (Pb)	Inorganic	Contaminated soil, Water, Air	Ingestion, Inhalation	1	10	Anemia, Neurological disorders, Developmental delays	Impaired cognitive functions, Reproductive issues	Krametter-Froetscher et al., (2007); Finnie et al., (2011); Patra et al., (2011); Okerefor et al., (2020); Slivinska et al., (2020); Mukherjee et al., (2022)
Mercury (Hg)	Organic, Inorganic	Coal combustion, Seafood	Ingestion, Inhalation	1.5	15	Neurological disorders, Tremors, Developmental delays	Growth retardation, Impaired vision, Kidney damage	Krametter-Froetscher et al., (2007); Ceccatelli et al., (2010); Bubber, (2019)
Cadmium (Cd)	Inorganic	Industrial waste, Fertilizers	Ingestion, Inhalation	0.5	5	Renal dysfunction, Osteoporosis, Gastrointestinal problems	Kidney damage, Bone demineralization, Cancer risk	Swarup and Dwivedi, (2002); Cai et al., (2009); Bilandzic et al., (2010); Gumasta et al., (2018); Wang et al., (2020)
Copper (Cu)	Inorganic	Waste water, Feed supplements, Copper-based fungicides	Ingestion	250	2,500	Liver damage, Gastrointestinal disturbances	Hemolytic anemia, Cirrhosis, Neurological issues	Elliott et al., (2020); Hinde, (2021); Quevedo et al., (2022)

2022). Metals like Cd and Pb inhibit androgen production, microtubule movement, and the expression of regulatory protein genes resulting in a drastic decline in sperm count and histopathological changes in the testis (Bhardwaj et al., 2021; Salihu et al., 2021). Metal exposure also initiates testicular damage by altering angiogenesis processes (Rzymiski et al., 2015). Cd exposure to Leydig cells increases cytokine production, inducing DNA breaks and reducing testosterone production leading to male infertility (Yang et al., 2022). Cd also stimulates α -adrenergic receptors producing excess glucocorticoids, inhibiting steroidogenesis and causing Sertoli cell damage (de Angelis et al., 2017; Slaby et al., 2019). A positive correlation between blood Cd levels and sperm abnormalities and an inverse relation with sperm density has been observed (Meligy et al., 2019).

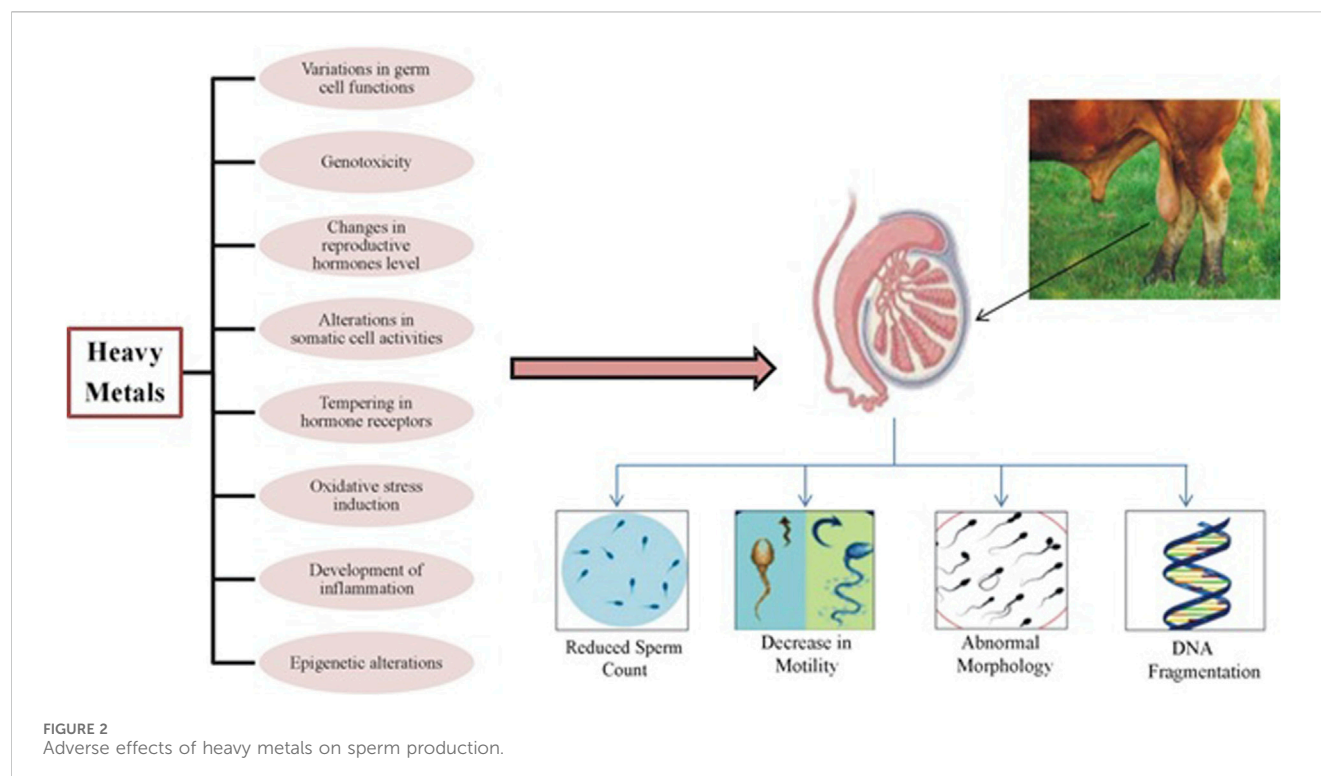
Blood-Testicular Barrier (BTB) disruption due to heavy metal toxicity distorts tight junctions that negatively influences spermatogenesis and increases sperm abnormalities (Mukherjee et al., 2022). DNA hypermethylation due to metal toxicity leads to the overexpression of DNMT3b causing malignancy of prostate epithelial cells and permanent infertility (Andelković et al., 2023). Further, metal toxicities may result in conditions like teratozoospermia, asthenozoospermia, reduced sperm surface mannose expression, loss of sperm head actin, acrosome reaction insufficiency, increased apoptosis, and altered transvascular fluid exchange with varicocele (Andelković et al., 2023). Varicocele affects semen quantity and quality by influencing sperm count, morphology, and motility (Ilieva et al., 2020). Certain other biochemical and histological changes are also associated with

heavy metals that adversely influence the reproductive performance of animals (Kar et al., 2015). Heavy metal in low concentration for a short period of time does not significantly affect reproductive performance of animals but moderate to high level in blood leads to poor semen quality and also reduces the reproductive hormones levels (Bhardwaj et al., 2021).

Toxic effects on female reproductive performance

The exposure of female animals to heavy metals affects oocyte maturation rate, germinal vesicle breakdown, and sex organ weight causing impairment in the reproductive process (Figure 3) (Fort et al., 2001; Dutta et al., 2022). During the embryonic development process, 17β -estradiol was found to synergistically modify the harmful effects of heavy metals on the female reproductive system (Chedrese et al., 2006). Metals like Cd disrupt the functions of the reproductive endocrine system, impairing oocyte release from graafian follicles, affecting ovulation, and ultimately impacting animal breeding (Fort et al., 2001; Dutta et al., 2022).

DNA damage induced by heavy metals leads to excessive ROS production, causing oocyte death during meiosis-I and reducing the number of oocytes entering Metaphase-II stage (Engwa et al., 2019). Chronic metal exposure inhibits the functional activity of the p450 gene that influences the morphology and physiology of ovarian granulosa cells (Chedrese et al., 2006). Heavy metals interfere with FSH production, binding with its receptor and



lower the rate of steroid synthesis in ovarian granulosa cells that affect steroidogenesis (Kirmizi et al., 2020).

Metal ions enter cells through L-type voltage-gated channels inhibiting Ca^{2+} ATPase pump activity and accelerating the expression of ERK, p38, and c-jun, crucial for granulosa cell proliferation and steroidogenesis (Sun et al., 2022). Some metals like Cd^{2+} replace Zn^{2+} in the regulatory domain of estrogen receptors (ERs) causing adverse changes in DNA binding domain elasticity and variations in steroidogenic gene expression at the transcriptional level (Paithankar et al., 2021). The development process of mammary glands is also adversely affected by the presence of metal ions inducing early arrival of puberty (Migdal et al., 2023).

Changes in estrogen and progesterone receptors due to heavy metal alterations act as causative agents for reproductive issues like spontaneous abortion, endometrial cancer, and estrogen-dependent diseases of mammary glands (Bhat et al., 2023). Metals like Cd can replace bivalent ions (Ca^{2+} and Zn^{2+}), and their deficiency during pregnancy increases the chances of spontaneous abortion. *In vitro* and *in vivo* studies show that Cd^{2+} exposure unfavorably influences oocyte maturation and causes chromosomal abnormalities (Leoni et al., 2002; Jia et al., 2011; Aglan et al., 2020).

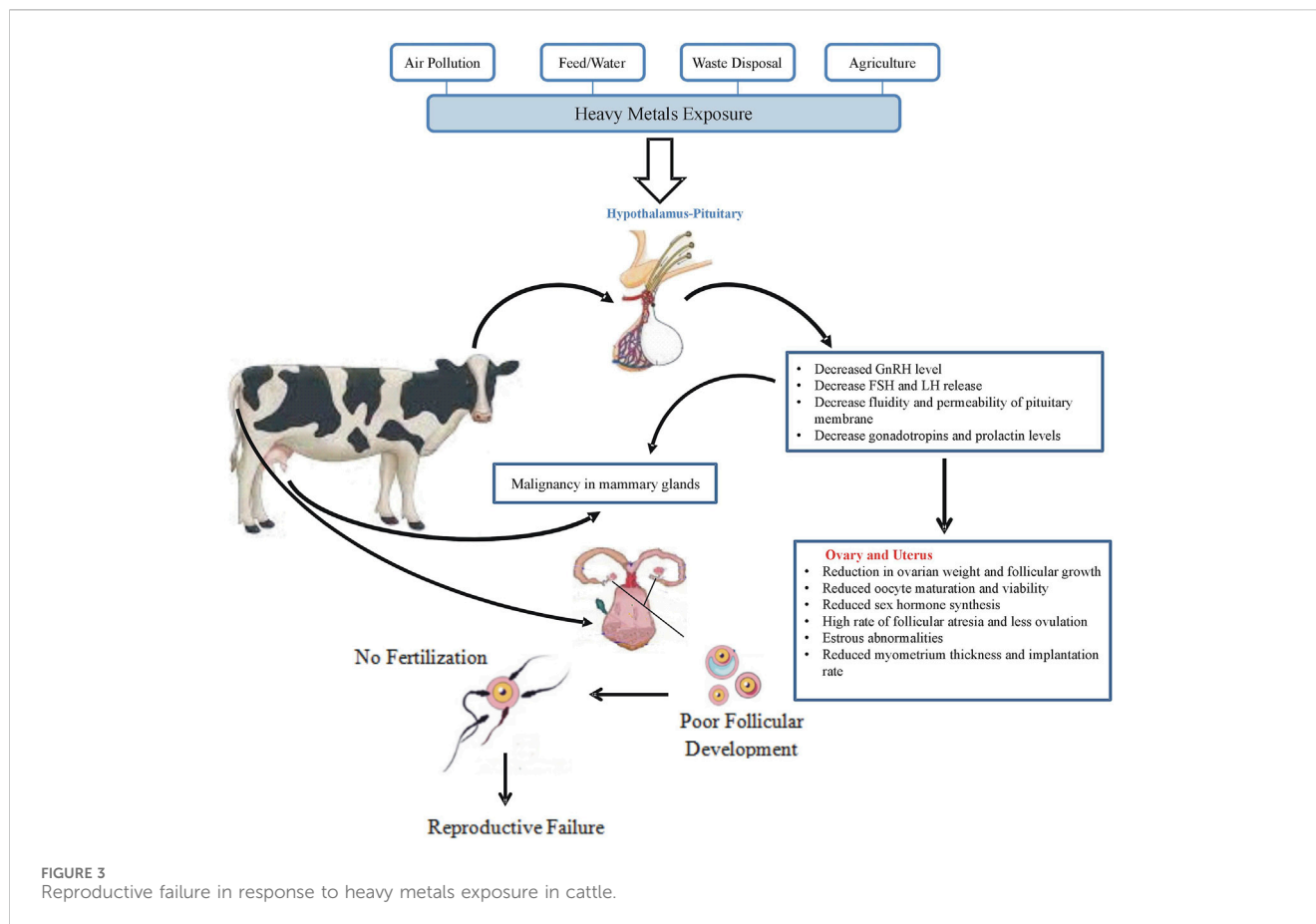
The influx of metals in females increases during nutritionally critical stages (pregnancy and lactation), decreasing essential mineral elements (Zn^{2+} , Fe^{2+} , Cu^{2+}) and promoting metal-induced reproductive toxicity. Metal exposure during pregnancy increases the chances of preterm labor, premature delivery, and low birth weight (Eaves and Fry, 2023). Tobacco smoke a source of metallo-toxins decreases female reproductive performance and causes structural changes in the fallopian tube and uterus (Semczuk and Semczuk-sikora, 2001).

Heavy metals can accumulate in the placenta of some animal species, affecting progesterone synthesis, inhibiting the transcription

of essential enzymes, and preventing the maintenance of pregnancy (Thompson and Bannigan, 2008; Caserta et al., 2013; Geng and Wang, 2019). *In vitro* administration of different heavy metals in endothelial cells promotes the synthesis of placental growth factor (PLGF) and vascular endothelial growth factor-A (VEGF-A) by mediating certain changes in mRNA expression. The transcription of the growth factors (VEGF-A and PLGF) alters the process of angiogenesis that is required for placental, implantation and embryogenesis (Yüksel et al., 2021). The defects in expression of VEGF-A and PLGF lead to lack of proper implantation of embryo, endothelial dysfunction, sub-infertility, pre-eclampsia and premature delivery (Rzymiski et al., 2015; Mishra and Bhardwaj, 2018).

Toxic metals and animal products quality

Heavy metals present a significant concern in the quality of animal products reared under commercial conditions as they are more susceptible to contamination from environmental pollution and industrial wastes (Hejna et al., 2019). While organic farming of animals emphasizes natural methods of production, including pasture-based systems and restricted use of synthetic inputs such as pesticides and fertilizers, organic animals are less likely to be exposed to heavy metals from industrial sources such as contaminated soil, water, and feed additives. Among heavy metals contamination in commercial production of animals, As stands out as a particular concern as livestock animals can experience its toxic effects through the consumption of contaminated feedstuffs. As is primarily excreted in the milk of exposed animals, while its residues have also been detected in chicken, duck, cow, and goat meat (Zahrana and Hendy, 2015).



As contamination deteriorates milk and meat quality that poses a serious risk to consumer health. Human exposure to As occurs through the consumption of contaminated foodstuff (milk and meat) as highlighted by a survey conducted by the European Food Safety Authority (EFSA) (Authority et al., 2021).

Despite the absence of a universally accepted safe limit for As in food, WHO has set an acceptable intake level of 3.0 g/kg BW for this toxic metal (Islam et al., 2014). The detection of As in cow's milk and poultry liver suggests them as preferred deposition sites compared to other meat parts (Das et al., 2021). Notably, milk contains inorganic As due to the inability of methylated As to cross the udder epithelium of cows. The highest accumulation of As occurs in casein (83%), while fat, whey protein and skimmed milk contain minute amounts of 10%, 4%, and 3% respectively. This highlights the complexity of metal distribution within dairy products (Das et al., 2021).

Moreover, milk can also be affected to some extent by Cr and Ni (Somasundaram et al., 2005; Ubwa et al., 2017). Some other potential sources of contamination include milk utensils, feed, and the animals' environment (Ismail et al., 2019). A study involving Jersey cows substantiated the bioaccumulation of toxic metals in milk with concentrations of Pb, Cd, Ni, and Cr reaching significant levels (Somasundaram et al., 2005). Interestingly, Pb concentrations exhibit an increasing pattern in the initial stages, followed by a stable level over time, contrasting with Cd levels that double after 10 days and remain constant thereafter (Somasundaram et al., 2005).

Feed additives and industrial emissions lead to varying heavy metal concentrations in the muscle tissue of pigs. A significantly

high concentration of Pb (0.05–0.58 mg kg⁻¹ d.m.) and Cd (0.02–0.04 mg kg⁻¹) has been reported in the muscles of fattening pigs (Medardus et al., 2014; Chalabis-Mazurek et al., 2021). Sheep reared under pasture and extensive systems accumulate Cd in kidneys and udder, while Pb, Zn and Cu are predominant in ribs, liver, and long bones (Abd-Elghany et al., 2020).

Poultry, especially from backyard rearing systems accumulate heavy metals from sources like landfills and contaminated soil, resulting in exceeding permissible limits in meat and eggs. Duck and goose reared near industrial areas show higher concentrations of As, Cd, and Hg in their eggs as compared to non-industrial sites (Korish and Attia, 2020; Kabeer et al., 2021). Bees serve as bioindicators of toxic metal contamination and retain a significant portion of these metals from their surrounding environment during honey processing (Bosancic et al., 2020). Horse muscles generally lack excess heavy metals, but kidneys and liver often accumulate Cd and Zn (Gupta et al., 2021). Wildlife in industrially exploited regions exhibits levels of heavy metals surpassing legal limits for human consumption, with wild boar being the most affected species (Verma et al., 2023).

Cytotoxic and oxidative effects of heavy metals

Pb exerts its cytotoxic effects by interfering with enzymes involved in heme synthesis, such as δ -aminolevulinic acid

dehydratase (ALAD) leading to anemia (Ebrahimi et al., 2023). Further, Pb disrupts calcium signaling pathways by interfering with calcium-dependent processes that affects neurotransmitter release and synaptic plasticity (Cory-Slechta et al., 2016). Hg impairs mitochondrial function and energy metabolism, causing cellular dysfunction and apoptosis (Ke et al., 2023). Moreover, by disrupting neurotransmitter signaling, Hg interferes with the function of ion channels and neurotransmitter transporters, contributing to neurotoxicity (Novo et al., 2021). Cd binds to sulfhydryl groups in proteins. This binding inhibits their activity and disrupting cellular functions (Genchi et al., 2020; Suhani et al., 2021). As toxicity inactivates enzymes involved in cellular metabolism, including those in the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (Machado-Neves and Spuza, 2023). As also disrupts cell cycle regulation, resulting in aberrant cell proliferation and apoptosis resistance (Naujokas et al., 2013).

Heavy metals induce the generation of highly reactive free radicals causing DNA damage, oxidation, protein depletion, lipid peroxidation, and various cytotoxic effects. The toxicity of different metals is primarily attributed to the production of ROS and RNS disrupting cellular redox potential. ROS with their high chemical reactivity produce free radicals like hydroxyl (OH), superoxide ($O_2^{\cdot-}$), alkoxyl (RO), and peroxy ($RO_2^{\cdot}$) as well as non-radicals such as H_2O_2 and peroxynitrite ($ONOO^-$) acting as oxidizing agents or converting into other radicals.

Intracellular, superoxide anion ($O_2^{\cdot-}$) generation involves redox components (e.g., semi-ubiquinone), enzymes (e.g., NADPH-oxidase (NOX), xanthine-oxidase), or auto-oxidation reactions (Brochin et al., 2008; Flora et al., 2012; Alkadi, 2020; Sylvester et al., 2022). Superoxide anion ($O_2^{\cdot-}$) exhibits limited reactivity under specific physiological conditions and cannot cross biological membranes. However, its reaction with nitric oxide (NO) leads to the production of peroxynitrite ($ONOO^-$) with highly reactive intermediates like the short-lived hydroxyl radical (OH) (Pastor et al., 2000).

Involvement of nitric oxide synthase isozymes, such as mitochondrial nitric oxide synthase (mtNOS) and endothelial nitric oxide synthase (eNOS) also result in the production of nitric oxide (NO) from L-arginine to citrulline. NO due to its amphipathic nature remains stable in anaerobic environments and can diffuse through plasma membranes and cytoplasm. Its interaction with superoxide anion results in the production of peroxynitrite ($ONOO^-$) (Szabo et al., 2007). An imbalance in ROS and RNS production or a decrease in scavenging activities as a consequence of external stimuli like metal exposure leads to alterations in cellular functions through direct changes in biomolecules and aberrant stimulation of specific signaling pathways that directly affect growth performance and production potential of animals.

Epigenetic modifications

Epigenetic modifications such as DNA methylation, DNA repair, histone modifications, transcription, RNA regulation, RNA stability, protein degradation, transposon activation, and gene copy number alterations are intricate processes influenced by pollutants containing heavy metals. These metals induce gene

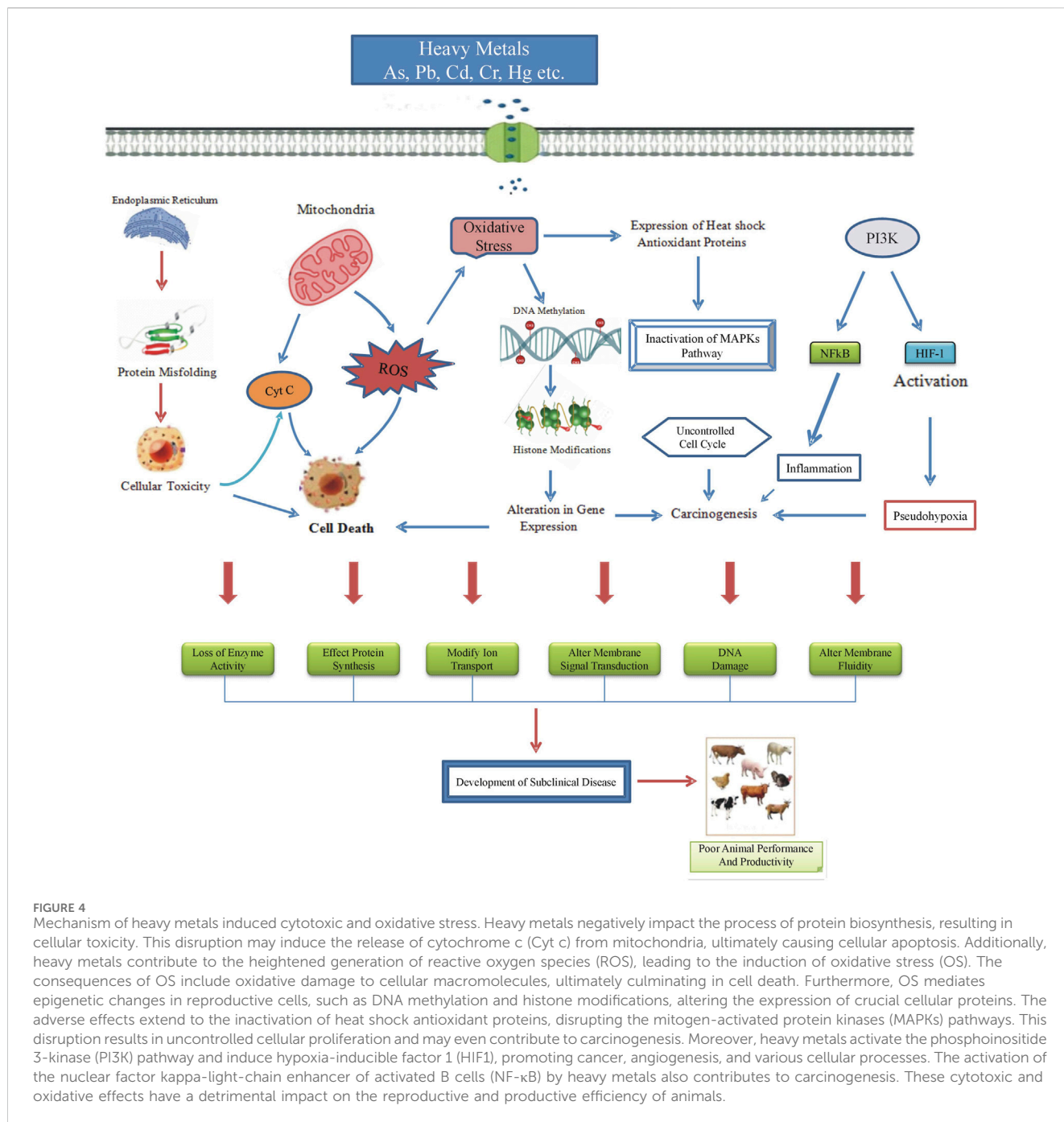
alterations, contributing to complex changes responsible for malignancies, allergic reactions, respiratory diseases, intrauterine growth inhibition, pre-eclampsia, and permanent infertility in affected animals (Edwards and Myers, 2007; Godfrey et al., 2016).

Current interest in epigenetics research and evidence of environmental variables influencing DNA methylation has expanded the study of health-related causes (Salnikow and Zhitkovich, 2008; Arita and Costa, 2009; Larson et al., 2010). Alteration in protein synthesis mechanisms due to heavy metal pollutants in the nucleus may lead to cellular toxicity, initiating the process of cell apoptosis (Figure 4) (Florea et al., 2005). In response to As, epigenetic modifications involve glutathione-reactive oxidation, increased expression of proteins (e.g., alpha-B-crystallin, heat-shock anti-oxidative stress proteins ferritin light chain) and enzymes (e.g., heme oxygenase-1, reductase, aldose), while glyceraldehyde-3-phosphate dehydrogenase activity is downregulated and extracellular signal-regulated kinases ERK-1 and ERK-2 are inactivated. These activities cause genomic damage and disrupt the cell cycle. DNA methylation is observed in the case of Cr and Cd exposure (Edwards and Myers, 2007; Arita and Costa, 2009). Trivalent Cr is essential in trace quantities for normal reproductive functions. While, it is chronic exposure leads to significant epigenetic alterations in sperm influencing parental imprinting, ovarian cyst formation, uterine anomalies, and tumors in male offspring. Trivalent As and bivalent Cd exposure affects cell associations in urothelial cells, lowering SPARC expression and playing a role in bladder tumor development (Larson et al., 2010). Prolonged Pb exposure increases superoxide dismutase (SOD) enzyme activity in neoplasia, suggesting a potential mechanism for reactive oxygen species production and carcinogenesis (Kasperczyk et al., 2004). These examples provide evidence that heavy metal exposures cause epigenetic alterations, linking heritable changes in gene expression to increased likelihood of various physiological disorders, including reproductive issues.

Monitoring of toxic metals contamination

Toxic metals stemming from escalating human activities necessitate increased monitoring due to their accumulation in the environment and animal tissues. This monitoring is crucial for estimating health hazards for both humans and animals as it involves identifying environmental contaminants and assessing affected areas (Khan et al., 2018). Estimating metal contents in animal tissues aids in diagnosing environmental pollution, accomplished through various biological techniques including the use of bio-indicators (Khan et al., 2019). Animal exposure to toxic metal poisoning is primarily influenced by contaminated feeds containing toxins like pesticides and metals (Khan et al., 2023). Monitoring animal feeds for toxic metal contamination is imperative especially in areas with polluted soils or industrial wastes (Usman et al., 2022).

The accumulation of toxic metals in the soil which enters the food chain through plants poses a direct threat to humans consuming animal products (Năstăsescu et al., 2020). Despite the accumulation of toxic metals in animal tissues, a lack of international standards for certain elements in food products poses risks to consumer health (European Commission, 2006).



Toxic metal bio-monitoring using blood plasma, urine, or hair samples offer insights into metal concentration and excretion patterns. Hg excretion occurs in urine and feces, while Pb is found in hair, blood, and urine samples (Hsieh et al., 2019). As can be detected in blood, feces, and keratin tissues hours after exposure. Diagnosis of toxic metal poisoning in cattle also involves testing milk, an indirect indicator of environmental pollution (Pšenková et al., 2020).

Toxic metals predominantly accumulate in internal organs making it feasible to establish standard levels in the body (Ke et al., 2023). Diagnosis and control of contamination in animal

feed ensure the safety of human food and health. High concentrations of Cd and Hg in animal feed (>10 ppm) are considered toxic, while Co, Cu, Pb, Mo, or Ba are regarded as toxic at concentrations above 40 ppm (Mc Geehan et al., 2020). Mandatory bio-monitoring programs should be established, requiring regular testing of blood, body fluids and tissue samples from livestock is essential, as relying solely on feed testing is insufficient for detecting metal contamination (McGeehan et al., 2020). These programs, overseen by veterinary authorities or certified laboratories, should set safe thresholds for heavy metal concentrations. Additionally, state

authorities must monitor and regulate industrial activities that contribute to metal contamination, implement education programs for livestock owners, and enforce penalties for non-compliance. Investing in research and fostering inter-agency collaboration will further strengthen efforts to prevent heavy metals toxicities in livestock environments.

Further, Collaborative efforts between veterinary, environmental, agricultural, and public health agencies are essential to address heavy metal contamination comprehensively. Public awareness campaigns should also be launched to educate farmers and the public about the risks of heavy metal contamination in livestock, while investments in testing facilities and laboratories will bolster monitoring efforts (Chen et al., 2022). Additionally, policy development and implementation, alongside surveillance systems to monitor contamination trends, are crucial for prompt response to outbreaks or incidents of toxicity. Through these measures, state authorities can safeguard livestock health and ensure the safety of the food supply chain.

Prevention and control of bioaccumulation of toxic metals

Soil remediation is used to reinstate the effectiveness of soil that indirectly reduces the chances of toxic metal exposure and their harmful effects (Xu et al., 2021). The techniques used for remediation of soil are based on biological and/or chemical protocols (Mahey et al., 2020). Bioremediation is used to clear water and soil from toxic metals that are harmful for living beings. This process utilizes a variety of plants and different microorganisms for biological restoration of contaminated areas. Soil can be reclaimed from Cu contents by using *Pseudomonas aeruginosa* and *Bacillus* spp. whereas phytoremediation is helpful in the clearance of Co from soil and water. The toxic metals are immobilized in plants, thus their further spread is prevented. The symbiotic bacteria of these plants possess the potential to adsorb these toxic metal pollutants. Chemical techniques for reduction of toxic metal pollutants reduce the bioavailability of metals (Singh and Prasad, 2015).

Environmental contamination with toxic metals extends to animal feed, leading to potential risks upon consumption (Lopez-Alonso et al., 2017). Contaminated animal feed introduces metals into animal bodies, eventually excreted in urine and feces (Hao et al., 2019). Animal dung rich in proteins and minerals may act as a fertilizer but can also be a source of toxic metal contaminants (Li et al., 2020). Composting reduces metal concentrations in manure by altering physicochemical properties and utilizing microorganisms to immobilize and oxidize metals (Hao et al., 2019; Wang et al., 2019). Landfills contribute to environmental pollution with toxic metals, generating leachate that contaminates soil and groundwater. Phytoremediation, using plants to extract and accumulate metals, aids in the recovery of landfill soils. Biochar produced during biomass pyrolysis reduces the bioavailability of toxic metals, improving soil quality. The deposition of sewage sludge in landfills poses a contamination risk but pyrolysis transforms sewage sludge into biochar, reducing metal spread and increasing soil pH (Penido et al., 2019; Elbehiry et al., 2020). The use of biochar

in sewage sludge treatment minimizes pollution risks and enhances soil fertility (Penido et al., 2019; Li et al., 2020).

Conclusion

Heavy metal toxicity has emerged as a major environmental challenge with detrimental impacts on livestock health and productivity. Heavy metals accumulate in the environment and then infiltrate animals from both natural and anthropogenic sources. Despite the essential role of some metals in maintaining biochemical and physiological functions, all metals exert their toxic effects via metabolic interference and mutagenesis. These metals affect various organs in animals, with the liver being typically the first impacted followed by the kidneys, brain, and reproductive system. The accumulation of heavy metals results in both gross and histopathological changes, ultimately leading to impaired growth and production. Prevention is paramount in addressing heavy metal contamination in livestock. Phytoremediation and intercropping can help to remove heavy metals from the environment before ingestion by animals. If heavy metals do enter the body, entero-absorbents can be employed to chelate them. Additionally, heavy metals commonly induce oxidative stress through various mechanisms, leading to altered redox potential, so including antioxidants in the therapeutic regimen has shown promise in significantly reducing the toxic effects associated with heavy metal exposure. Moreover, upcoming research will benefit from evaluating innovative targets as protective measures against organ toxicity caused by heavy metals.

Author contributions

AA: Writing–review and editing, Writing–original draft, Software, Methodology, Investigation, Formal Analysis, Data curation, Conceptualization. NM: Writing–review and editing, Software, Methodology.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Conflict of interest

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OPEN ACCESS

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RECEIVED 30 May 2024

ACCEPTED 24 June 2024

PUBLISHED 12 July 2024

CITATION

Shi Y, Gao Z, Xu B, Mao J, Wang Y, Liu Z and Wang J (2024), Protective effect of naringenin on cadmium chloride-induced renal injury via alleviating oxidative stress, endoplasmic reticulum stress, and autophagy in chickens. *Front. Pharmacol.* 15:1440877. doi: 10.3389/fphar.2024.1440877

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Protective effect of naringenin on cadmium chloride-induced renal injury via alleviating oxidative stress, endoplasmic reticulum stress, and autophagy in chickens

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Cadmium (Cd) is a highly hazardous toxic substance that can cause serious harm to animals. Previous studies have indicated that cadmium chloride (CdCl₂) can damage organs, such as the liver, ovaries, and testicles. Naringenin (Nar) represents a flavonoid with various properties that promote the alleviation of Cd-induced damage. In this experiment, 60 chickens were divided into the control group, 150 mg/kg CdCl₂ treatment group, 250 mg/kg Nar treatment group, and 150 mg/kg CdCl₂ + 250 mg/kg Nar co-treatment group, which were treated for 8 weeks. Kidney tissues samples were collected to investigate kidney function, including oxidative stress (OS), endoplasmic reticulum (ER) stress, and autophagy activity. Experimental results showed the decreased weight of chickens and increased relative weight of their kidneys after CdCl₂ treatment. The increase in NAG, BUN, Cr, and UA activities, as well as the increase in MDA and GSH contents, and the decrease activities of T-AOC, SOD, and CAT in the kidney, manifested renal injury by OS in the chickens. TUNEL staining revealed that CdCl₂ induced apoptosis in renal cells. CdCl₂ upregulates the mRNA and protein expression levels of GRP78, PERK, eIF2α, ATF4, ATF6, CHOP, and LC3, and inhibited the mRNA and protein expression levels of P62 proteins, which leads to ER stress and autophagy. The CdCl₂ + Nar co-treatment group exhibited alleviated CdCl₂-induced kidney injury, OS, ER stress, and autophagy. Research has demonstrated that Nar reduces CdCl₂-induced kidney injury through alleviation of OS, ER stress, and autophagy.

KEYWORDS

cadmium chloride, naringenin, oxidative stress, endoplasmic reticulum stress, autophagy

1 Introduction

Cadmium (Cd) is a prevalent environmental pollutant. The sources of Cd are very diverse, including industrial and agricultural activities, such as lead and zinc mines, nonferrous metal smelting, battery production, electroplating, plastics manufacturing, and pesticide production (Al Olayan et al., 2020; Charkiewicz et al., 2023). Human activities are also the main sources of Cd, such as copper and nickel smelting, burning of fossil fuels, phosphate-fertilizer production, and other activities that may lead to Cd release (Nna et al., 2017). The main exposure route of Cd is through respiratory inhalation,

followed by food and water intake, with skin absorption being the least common. Once Cd enters the body, it is transported through the bloodstream to various organs, where it accumulates in the kidneys and liver. Its elimination from the body is relatively slow (Genchi et al., 2020). Cd adversely affects human and animal health, and it can damage the liver and kidney tissues of animals (Ali et al., 2022). Cd and its compounds are also recognized as human carcinogens. Their effects can lead to various types of cancer, such as breast cancer, gastrointestinal tumor, lung cancer, and prostate cancer (Huff et al., 2007).

The kidneys are a vital component of the urinary system, playing a crucial role in filtering impurities from the blood and regulating fluid balance (Osborn et al., 2021). Research indicated that the kidneys are one of the main organs affected by Cd exposure (Chen et al., 2019). Poultry is an important source of animal protein in the human diet. The European Commission has set a maximum safe level of Cd in poultry kidneys at 1.0 mg/kg (Commission Regulation, 2024). However, environmental factors such as feed, soil, and water contaminated with high concentrations of Cd may pose a threat to poultry health (Aljohani, 2023). Cd can also be gradually absorbed by the human body through the consumption of Cd-contaminated plants and animals, thereby posing a potential health hazard. Therefore, reducing the impact of Cd exposure on poultry, particularly chickens, has great significance.

Cd-induced oxidative stress (OS) serves as the primary cause of kidney injury (Ge et al., 2019). OS is an imbalance in the body's oxidative and antioxidant systems caused by harmful stimuli or the removal of senescent cells from the body. Antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and non-enzymatic compounds such as glutathione (GSH). They are endogenous antioxidants in the body and play a crucial role in maintaining redox homeostasis in body cells. Malondialdehyde (MDA) is the product of lipid peroxidation. By measuring MDA content, it can be assessed the extent of peroxidation damage in tissues. It can also indirectly indicate the extent of tissue peroxidation damage. Total antioxidant capacity (T-AOC) comprises numerous antioxidant substances and enzymes. It can be utilized to evaluate antioxidant capacity in kidney tissues. Cd induces OS by increasing the level of the oxidative product MDA, which inhibits the antioxidant enzyme activity and depletes the GSH activity (Ge et al., 2019). Zhuang et al. (2019) found that Cd led to elevated MDA levels in duck kidney tubular epithelial cells and decreased the activities of CAT, SOD, T-AOC, and GSH, which resulted in OS.

Endoplasmic reticulum (ER) stress plays an important role in organismal toxicity induced by Cd (Chen et al., 2019; Wang et al., 2023). Under stimulation, the ER struggles to bear the load of folded or misfolded proteins, which leads ER stress. Glucose-regulating protein 78 (GRP78) is an important regulator of ER stress. During ER homeostatic equilibrium, GRP78 binds to ER stress receptors, including protein kinase RNA-like ER kinase (PERK), inositol-requiring protein 1 (IRE1), and activating transcription factor 6 (ATF6), which causes the inhibition of the unfolded protein response (UPR). By contrast, during ER stress, GRP78 bind to misfolded proteins and separates from ER stress receptors (Kawaguchi et al., 2020). This action activates eukaryotic translation initiation factor 2 (eIF2 α), transcription factor 4

(ATF4), and signaling molecules downstream of the C/EBP homologous protein (CHOP). Multiple researchers have found that Cd induces ER stress after exposure to the testes, heart, and ovaries (Wan et al., 2018; Wang et al., 2023; Zhu et al., 2024).

Autophagy is a complex process of degrading harmful components within cells through lysosomes and is considered a form of programmed cell death. Autophagy can be divided into macroautophagy, chaperone-mediated autophagy, and microautophagy. The most common is macroautophagy, so it is often considered a representative of autophagy. The core process of autophagy is the formation of autophagosomes, which is regulated by autophagy-related genes (Atg) and has great significance (Nishimura and Tooze, 2020). Studies have shown that Cd can inflict organ damage through autophagy (Ma et al., 2022).

Flavonoids are natural organic compounds that exist in nature and have antioxidant, antibacterial, anti-inflammatory, and myocardial protective effects (Chen et al., 2023). Naringenin (Nar) is a dihydroflavonoid extensively found in citrus plants. It exerts pharmacological effects such as antioxidation, anti-inflammation, anti-injury, and kidney protection and is widely used in food and medicine (Wang et al., 2012; Wang et al., 2017). The antioxidant activity of Nar is primarily demonstrated in scavenging free radicals, preventing lipid peroxidation, and enhancing the body's antioxidant activity by reducing reactive oxygen species (ROS) production (Lu et al., 2022). Most studies have shown that Nar has a kidney-protective effect. Nar can alleviate the kidney damage caused by CCl₄ in rats and ease the kidney damage in rats with gestational diabetes mellitus (Zhao et al., 2020; Ammar et al., 2022). However, the research on the amelioration of cadmium chloride (CdCl₂)-induced kidney injury by Nar in chickens is limited.

CdCl₂ tends to accumulate in living organisms, so it poses a hazard to the poultry industry and to humans who consume Cd-contaminated poultry. Thus, mitigating the associated risks is essential. Nar, as a natural organic compound, can alleviate the damage caused by Cd through its biological activity. In the present study, CdCl₂ and Nar were supplemented into diets to investigate the effects of Nar on CdCl₂-induced renal injury in chickens and its mechanisms, including OS, ER stress, and autophagy.

2 Materials and methods

2.1 Chemicals and reagents

Cadmium chloride (CdCl₂, 99.95% purity) (CAS No. 7790-78-5, Molecular weight: 228.36) and Naringenin (Nar, 97% purity) (CAS No. 67604-48-2, Molecular weight: 272.26) were procured from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Uric acid (UA, C012-2-1), Blood Urea Nitrogen (BUN, A013-2-1), Creatinine (CAT, A007-1-1), β -N-Acetylglucosaminidase (NAG, A031-1-1), Superoxide Dismutase (SOD, A001-2-2), Catalase (CAT, A007-1-1), Glutathione (GSH, A006-2-1), Total antioxidant capacity (T-AOC, A015-3-1) and Malondialdehyde (MDA, A003-1-2) kits were selected from Nanjing Jiancheng Institute of Biological Engineering (Nanjing, China).

TABLE 1 Nutrient content of chickens standard diet.

Nutrients	Concentrations (%)
Crude protein ($\geq$)	16.0
Crude fibre ($\leq$)	8.0
Crude ash ($\leq$)	15.0
Calcium	3.3–4.5
Total phosphorus ($\geq$)	0.4
Sodium chloride	0.3–0.8
Lysine ($\geq$)	0.85
Methionine	0.3–0.9
Moisture ($\leq$)	14.0

2.2 Animal experiment

Sixty 1-day-old male Hy-Line White chickens that passed quality inspection were evenly divided into the control group (standard diet), 150 mg/kg CdCl₂ group (standard diet supplemented with CdCl₂), 250 mg/kg Nar group (standard diet supplemented with Nar), and 150 mg/kg CdCl₂ + 250 mg/kg Nar co-treatment group (standard diet supplemented with CdCl₂ and Nar), to establish an animal model of chronic Cd poisoning. Each group of 15 animals was kept in cages with ample space for movement, suitable temperature, full sunlight, and fresh air (Size: 160 cm × 80 cm × 40 cm). They had free access to feed and water. The standard diet used in the experiment was purchased from Mengjin County Fanda Feed Factory in China (Table 1 shows the nutritional content). The applied doses of CdCl₂ and Nar were based on previous preexperiments. After 8 weeks of treatment, the chickens were euthanized (exsanguination following ether anesthesia), collect neck blood. The abdominal cavity was opened with sterile scissors to remove the kidneys, which is located on both sides of the sacrum and in the kidney fossa of the ilium.

2.3 Body weight and relative kidney weight

Chickens were weighed weekly, and their weight data were recorded. At the end of the test, the chickens were euthanized, the kidneys were removed, weighed, and recorded.

$$\text{Relative kidney weight} = \text{Kidney weight} / \text{Chicken weight} \times 100\%$$

2.4 Determination of kidney function and antioxidant indexes

Blood was collected from the necks of chickens and stored in test tubes. Then, the blood samples were centrifuged at 3,000 rpm and 4°C, and the serum was collected. Kidney function indexes, such as Uric acid (UA), Blood urea nitrogen (BUN), Creatinine (Cr), and β -N-acetyl-glucosaminidase assay kit (NAG), were measured. A total of 0.1 g kidney tissue was collected and added with nine times normal saline. Afterward, the kidney tissue was ground at a low temperature for 90 s and centrifuged at 3,000 rpm and 4°C for

10 min. The supernatant was collected to detect antioxidant indexes, including CAT, T-AOC, MDA, SOD, and GSH. All the necessary kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Among them, UA, Cr, T-AOC, MDA, and GSH were determined by the enzyme-labeling method by through a Tecan instrument (model: Infinite M Nano, Swiss Confederation). BUN, NAG, CAT, and SOD levels were determined using a spectrophotometer with MAPADA (model: UA-1800PC, Shanghai).

2.5 Pathological section analysis of kidney

Pathological sections of the kidney were stained with hematoxylin-eosin staining (H&E). A small piece of kidney tissue was cut into 4 μ m tissue blocks and fixed in 4% paraformaldehyde for 24 h. After rinsing with running water three times for 5 min each time, the samples were dehydrated with ethanol to varying concentrations for 45 min (30%, 50%, 70%, 95%, and 100%). The tissue blocks were initially immersed in a mixture of ethanol and xylene, followed by pure xylene to render the kidney tissue transparent. After paraffin embedding, and the tissue sections embedded were transferred onto slides at 60°C and dried overnight using a microtome. The treated sections were then dewaxed and rehydrated, colored with H&E staining solution, and observed a microscopy.

2.6 Detection of renal-cell apoptosis by TUNEL method

The prepared kidney paraffin sections were soaked in xylene twice for 5 min each time. They were then treated with gradient ethanol once for 3 min each time. The tissues were then treated with Proteinase K for 30 min and washed with PBS. After preparing the TUNEL reaction mixture, 50 μ L was added to the specimen, the mouth film was sealed in a dark, wet box, and the reaction was carried out at 37°C for 60 min. After adding to the termination reaction buffer and rinsing three times with PBS, DAB substrate was added to the specimen, reacted at 25°C for 10 min, and rinsed. Hematoxylin was redyed and washed with distilled water. After gradient dehydration in alcohol and xylene, the sample was sealed for transparency. Apoptotic cells were observed using an optical microscope and photographed.

2.7 Quantitative real-time PCR (RT-qPCR) detection

The kidney tissue, frozen at –80°C, was cut into pieces the size of mung beans. They were then placed into a 1.5 mL enzyme-free tube containing 800 μ L of TRIgene (Genstar, Beijing) and ground using a homogenizer for 60 s. After adding 200 μ L of chloroform, mixing well, and centrifuging for 15 min, the supernatant was collected. An equal amount of isopropyl alcohol was added, centrifugation was performed again for 10 min, and the supernatant was discarded. We added 75% anhydrous ethanol to the samples, washed them twice, and centrifuged them for 15 min each time. Anhydrous ethanol was

TABLE 2 Fluorescent quantitative PCR primer sequence.

Gene	Target gene sequences (5' → 3')	TM (°C)	Product length	Gen bank No.
GRP78	F: ATCAGCCCACTGTGACCATT R: AGCAGGAGGGATTCCAGTCA	F = 56.6 R = 58.3	101bp	XM_046928788.1
PERK	F: TCATCCAGCCTCAGTAAACC R: ACAACATCCTCGCCCAGT	F = 54.2 R = 56.8	164bp	XM_040671515.2
eIF2α	F: CCCAGTTTCACAGAGAGCG R: CCTGCTGTGCCATCTTTG	F = 56.2 R = 54.4	248bp	XM_046923980.1
ATF4	F: GTTTTCAAGGCACCGCACA R: ACTTTCCTCAGCCACCGAAC	F = 56.9 R = 57.9	176bp	XM_046906693.1
CHOP	F: GGCCTGGTTCAATATGGGGA R: AATGTCTGCATAGGACACTGGT	F = 57.5 R = 55.8	182bp	XM_046934870.1
ATF6	F: TAGCAAGGAGCCCAGCC R: GCCTTTGTGACTCTGTTCG	F = 59.0 R = 59.8	185bp	XM_040677276.2
LC3	F: GACTTGTAGAGGCTTTCCTTCATAG R: GACTTCATCTTCAGCAGAACACTAC	F = 54.7 R = 55.2	191bp	NM_001006279.3
P62	F: GCGGCGAGAAGGAGATG R: GCAGCAGGGGCAGTCAA	F = 56.6 R = 59.4	194bp	XM_040682727.2
Beclin-1	F: AAGTGTGGAGAACCAGATGCG R: GCAAGGGCATGCAGTAACAG	F = 57.7 R = 57.3	210bp	NM_001006332.1
ATG5	F: AGAGATGTGTGGTTTGGACGC R: GCCGAGGAAGGGCTGTATT	F = 57.9 R = 57.8	271bp	XM_046914044.1
β-actin	F: CCGCTCTATGAAGGCTACGC R: CTCTCGGCTGTGGTGGTGAA	F = 58.6 R = 60.3	128bp	NM_205518.2

removed by suction, the RNA was dried, and 30 μL of enzyme-free water was added to mix thoroughly for the determination of nucleic acid concentration. The proposed RNA was reverse transcribed into cDNA by using StarScript III All-in-one RT Mix with gDNA Remover kit (Genstar, Beijing). Finally, according to the reaction system (20 μL) and conditions specified in the RT-qPCR instructions, three techniques were repeated for each gene. Using β-actin as the internal reference, BIO-RAD fluorescence quantitative PCR (model: C1000 Touch™ Thermal Cycle, United States) was used to obtain the results. The expression of target genes was calculated by the $2^{-\Delta\Delta Ct}$ method (Table 2).

2.8 Western blot

The lysate was prepared using phosphatase inhibitor mixture A (Cat: No. P1081, Beyotime), protease inhibitor mixture (Cat: No. P1010, Beyotime) and RIPA lysate (strong) (Cat: G2002, Servicebio). A total of 400 μL prepared lysate was added to 40 mg kidney tissue for complete cleavage and protein extraction. A BCA kit was used in the assessment of protein content. The protein was denatured, added to the prepared 12% gel and then transferred to a 0.45 μm PVDF membrane (4 cm × 8.5 cm) using SDS-PAGE electrophoresis. Skimmed milk powder was incubated, then the appropriate concentration of primary antibodies was selected: LC3 (1:1,000, 2775S, Cell Signaling), GRP78 (1:6,000, 11587-1-AP, Proteintech), PERK (1:500, 24390-1-AP, Proteintech), CHOP (1:1,500, 15204-1-AP, Proteintech), ATF4 (1:800, 10835-1-AP, Proteintech), ATF6 (1: 5,000, 24169-1-AP, Proteintech), eIF2α (1:1,000, ab169528, Abcam), P62 (1:1,000, PM066, MBL), Beclin-1 (1:4,000, 11306-1-AP,

Proteintech), and ATG5 (1:1,000, 10181-2-AP, Proteintech) for 12–18 h. The secondary antibody was incubated for 50 min, and washed with TBST three times in between. Aplegen chemiluminescence instrument (Model: Omega Lum G, United States) was utilized for exposure and analysis of gray values.

2.9 Statistical analysis

SPSS 26.0 was used to analyze the differences in experimental results (one-way ANOVA and LSD), and GraphPad Prism 8 was used for to plot data. A *P*-value less than 0.05 was considered statistically significant (*P* < 0.05). Values are expressed as the mean ± SEM.

3 Results

3.1 Effect of Nar and CdCl₂ on chicken weight

Nar showed no significant effect on the weights of chickens (Table 3). Compared with the control group, the body weights of chickens exposed to CdCl₂ exhibited significant differences starting from the fourth week, with decreases of 41.49%, 40.24%, 41.07%, 40.86%, and 40.58% (*P* < 0.01). However, compared with the CdCl₂ group, the body weights of CdCl₂ + Nar co-treatment group increased by 13.51%, 12.51%, 13.80%, 16.15%, and 18.28% (*P* < 0.05) from week 4. Nar reduced CdCl₂-induced weight loss in chickens, and this effect gradually increased over time.

TABLE 3 Effect of Nar and CdCl₂ on the body weight of chickens.

Days of treatment	Control	CdCl ₂	Nar	CdCl ₂ + Nar
0 week	49.78 ± 4.41	50.4 ± 1.46	48.18 ± 3.2	49.13 ± 1.44
1 week	107.98 ± 8.36	77.02 ± 5.97	106.15 ± 10.63	91.8 ± 12.69
2 weeks	191.77 ± 21.77	147.02 ± 15.94	200.18 ± 22.59	169.82 ± 17.95
3 weeks	289.43 ± 9.68	189.33 ± 21.69	309.02 ± 23.78	211.17 ± 37.54
4 weeks	459.3 ± 13.19	268.72 ± 47.7**	461 ± 12.26	305.03 ± 16.58 [#]
5 weeks	632.83 ± 19.85	378.17 ± 52.66**	634.83 ± 26.08	425.5 ± 33.54 [#]
6 weeks	840 ± 17.89	495 ± 63.8**	828.33 ± 29.27	563.33 ± 55.02 [#]
7 weeks	1011.67 ± 43.09	598.33 ± 96.21**	1008.33 ± 37.64	695 ± 75.03 [#]
8 weeks	1211.67 ± 56.01	720 ± 101.39**	1176.67 ± 57.15	851.67 ± 51.15**

Values are expressed as the mean ± SEM. n = 6.
P < 0.01: compared with the control group.
P < 0.05,
P < 0.01: compared with the CdCl₂ group.

TABLE 4 Relative kidney weight results after 8 weeks of treatment.

Treatment	Relative kidney weight
Control group	0.6 ± 0.06
CdCl ₂ group	1.18 ± 0.19**
Nar group	0.59 ± 0.07
CdCl ₂ + Nar group	0.98 ± 0.15 [#]

Values are expressed as the mean ± SEM. n = 6.
P < 0.01: compared with the control group.
P < 0.05: compared with the CdCl₂ group.

3.2 Effect of CdCl₂ and Nar on kidney weight of chickens

Detection of the relative weight of chicken kidney organs (Table 4). The relative kidney weight of CdCl₂ was significantly higher than that of the control group, showed an increase of 96.67% (*P* < 0.01). Compared with the CdCl₂ group, the CdCl₂ + Nar co-treatment group presented significantly reduced relative kidney weight of chickens by 16.95% (*P* < 0.05).

3.3 Effects of CdCl₂ and Nar on kidney function in chickens

The kidney function indicators UA, BUN, CRE, and NAG were tested in the investigation of the effect of CdCl₂ on kidney damage in chickens (Figure 1). Compared with the control group, the levels and activities of serum UA (312.11%), BUN (140.04%), Cr (215.02%), and NAG (55.27%) in the Cd group were significantly increased (*P* < 0.01). The levels and activities of UA (44.55%), BUN (27.64%), Cr (36.66%), and NAG (18.71%) in the serum of chickens from the CdCl₂ + Nar co-treatment group were significantly lower (*P* < 0.05) than those in the CdCl₂ group. This study indicates that Nar alleviated CdCl₂-induced kidney impairment in chickens.

3.4 Effects of CdCl₂ and Nar on kidney oxidation indexes in chickens

Antioxidant indexes, including CAT, T-AOC, MDA, SOD, and GSH, were detected in the assessment of Cd-induced OS in the kidneys (Figure 2). Compared with the control group, the levels of T-AOC (20.51%), CAT (35.05%), and SOD (27.94%) of the CdCl₂ group were significantly decreased, whereas the levels of GSH (241.01%) and MDA (120.05%) were significantly increased (*P* < 0.01). In addition, compared with the CdCl₂ group, significantly higher activities of T-AOC (9.58%), CAT (27.36%), and SOD (26.20%) were observed in the CdCl₂ + Nar co-treatment group, and the levels of GSH (26.04%) and MDA (26.58%) were significantly decreased (*P* < 0.05). Nar alleviated CdCl₂-induced OS in chicken kidneys through its antioxidant effect.

3.5 Observation of a kidney pathological section

Observations of pathological section showed that the control group (Figure 3A) and Nar group (Figure 3C) had clear renal-tubule boundaries and normal glomerular structure. Renal injury was severe in the CdCl₂ group (Figure 3B), with blurred and irregular glomerular boundaries (red arrow), as well as dissolved nucleus (blue arrow). The morphology of the renal tubules was incomplete, and the boundary before the renal tubules was unclear (black arrow). In the CdCl₂ + Nar group (Figure 3D), renal tissue injury significantly improved.

3.6 Renal cell apoptosis was detected using the TUNEL method

To verify the effects of CdCl₂ and Nar on the apoptosis of chicken renal cells, TUNEL staining was used to detect apoptosis in each group (Figure 4). The green fluorescence intensity of the control group (Figure 4A) and Nar group (Figure 4C) was

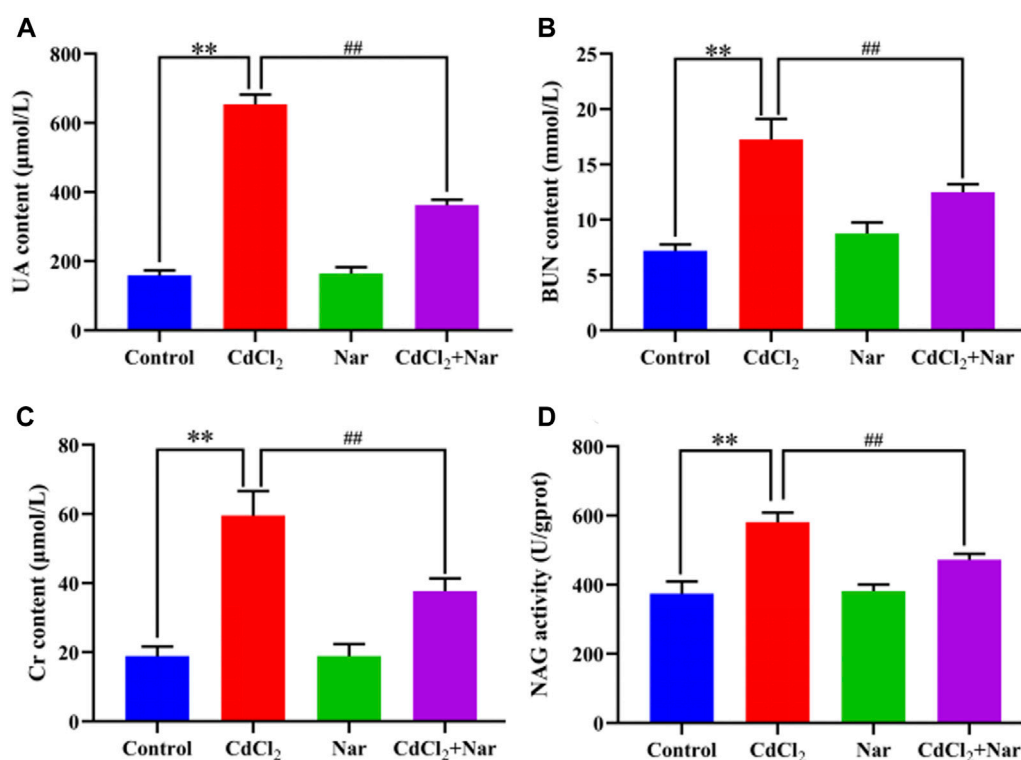


FIGURE 1
Effects of CdCl₂ and Nar on kidney function in chickens. (A) UA, (B) BUN, (C) Cr, (D) NAG. n = 6. Values are expressed as the mean ± SEM. **P < 0.01: compared with the control group. ##P < 0.01: compared with the CdCl₂ group.

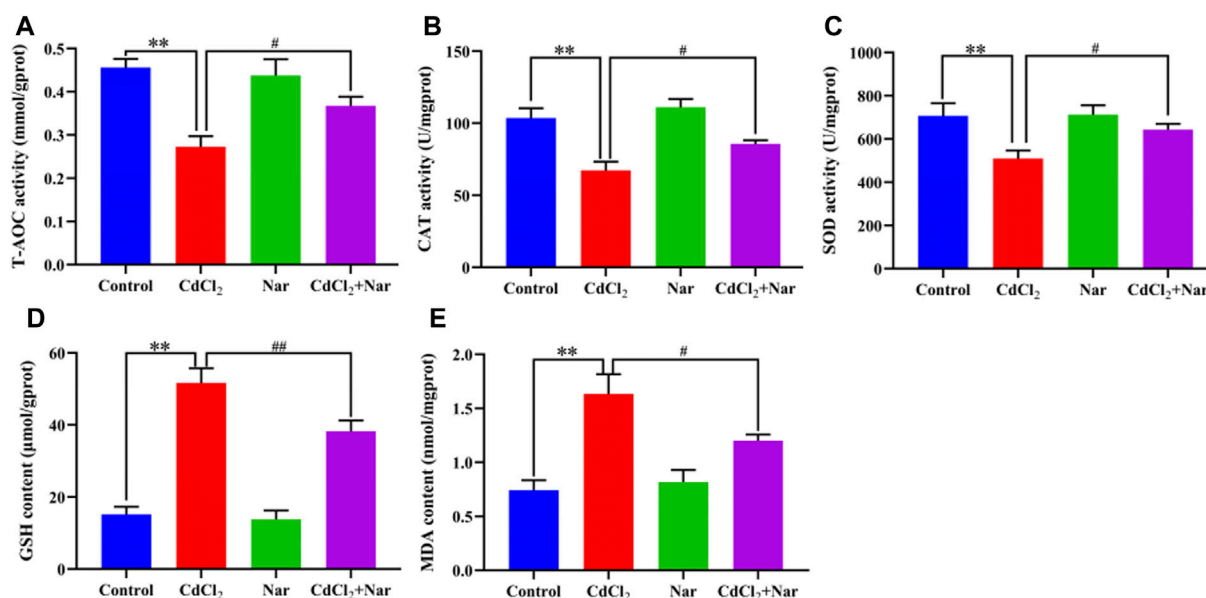


FIGURE 2
Effects of CdCl₂ and Nar on kidney antioxidant levels in chickens. (A) T-AOC, (B) CAT, (C) SOD, (D) GSH, (E) MDA. n = 6. Values are expressed as the mean ± SEM. **P < 0.01: compared with the control group. #P < 0.05, ##P < 0.01: compared with the CdCl₂ group.

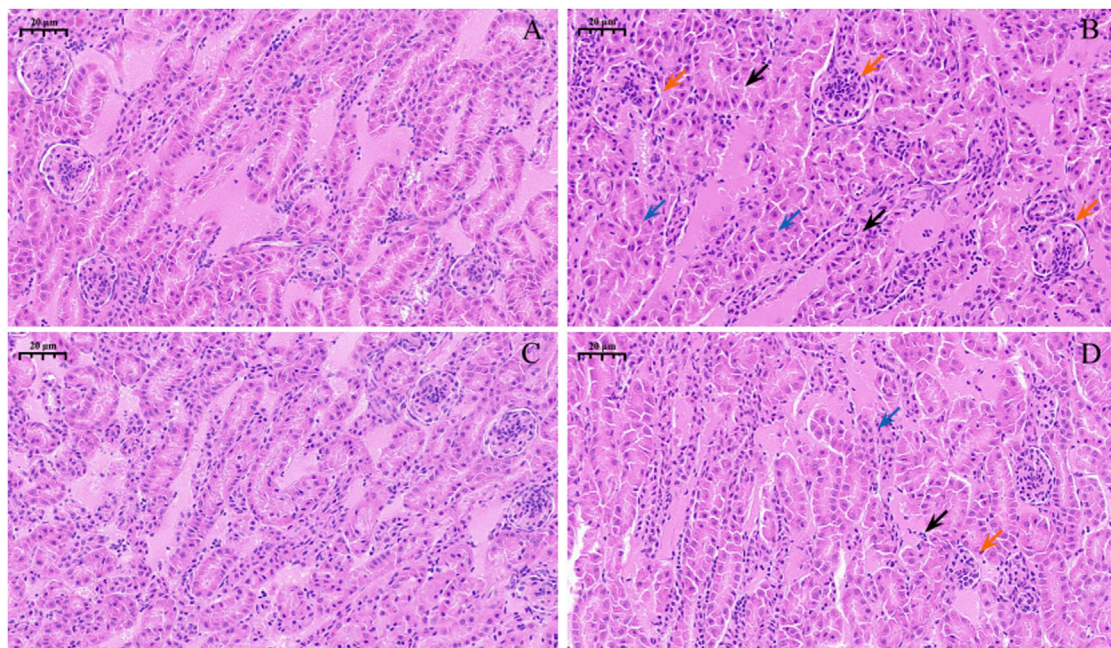


FIGURE 3

Histopathological observation of Nar intervention on the kidneys of chickens with chronic CdCl_2 poisoning. (A) control group, (B) CdCl_2 group, (C) Nar group, (D) CdCl_2 + Nar group. The scale is 20 μm . Red arrow: blurred and irregular glomerular boundaries; blue arrow: nucleus is dissolved; black arrow: boundary before the renal tubules is unclear.

similar, but the difference was not significant ($P > 0.05$). However, in the CdCl_2 group (Figure 4B), the green fluorescence intensity was significantly enhanced compared with those of control group and Nar group ($P < 0.01$). In the CdCl_2 + Nar group (Figure 4D), the green fluorescence intensity was significantly weaker than that in the CdCl_2 group ($P < 0.01$). Analysis of TUNEL-positive fluorescence intensity in each group (Figure 4E).

3.7 Effects of CdCl_2 and Nar on mRNA expression levels of ER stress-related genes in chicken kidneys

We tested the mRNA expression levels of ER stress-related genes, to verify the effects of CdCl_2 and Nar on ER stress (Figure 5). Results showed that the mRNA expression levels of GRP78 (96.68%), PERK (213.24%), ATF6 (85.61%), eIF2 α (156.34%), ATF4 (61.74%), and CHOP (82.9%) in the CdCl_2 group was significantly higher than that in the control group ($P < 0.01$). The mRNA expression levels of the GRP78 (26.9%), PERK (41.08%), ATF6 (27.41%), eIF2 α (33.49%), ATF4 (19.74%), and CHOP (23.22%) in the CdCl_2 + Nar group was significantly lower than that in the CdCl_2 group ($P < 0.01$). No significant difference existed between the control group and Nar group ($P > 0.05$).

3.8 Effects of CdCl_2 and Nar on ER stress-related proteins in chicken kidneys

To further verify the effects of CdCl_2 and Nar on ER stress, we examined the expression levels of ER stress-related proteins

(Figure 6). Results showed that the proteins expression levels of GRP78 (18.02%), PERK (55.05%), ATF6 (72.18%), eIF2 α (36.09%), ATF4 (41.12%), and CHOP (51.86%) in the CdCl_2 group were significantly higher than those in the control ($P < 0.01$). The proteins expression levels of GRP78 (11.91%), PERK (25.14%), ATF6 (22.23%), eIF2 α (7.23%), ATF4 (16.34%), and CHOP (22.31%) in the CdCl_2 + Nar co-treatment group was significantly lower than that in the CdCl_2 group ($P < 0.01$). The results indicate that CdCl_2 activates ER stress in chicken kidneys, whereas Nar alleviates CdCl_2 -induced ER stress.

3.9 Effects of CdCl_2 and Nar on mRNA expression levels of autophagy-related genes in chicken kidneys

The mRNA expression levels of autophagy genes such as Microtubule-associated protein light chain 3 (LC3), Sequestosome 1 (P62), Beclin-1, and Autophagy-related gene 5 (ATG5) were detected to verify the effects of CdCl_2 and Nar on autophagy (Figure 7). Results showed that the mRNA expression levels of the autophagy genes LC3 (90.49%), Beclin-1 (58.12%), and ATG5 (77.21%) in the CdCl_2 group were significantly higher than those in the control group ($P < 0.01$). Additionally, the mRNA expression level of P62 (48.02%) were significantly decreased ($P < 0.01$). Conversely, compared with the CdCl_2 group, the mRNA expression levels of autophagy genes LC3 (23.05%), Beclin-1 (18.74%), and ATG5 (25.73%) in the CdCl_2 + Nar group decreased, whereas the expression level of P62 (57.31%) was significantly increased ($P < 0.05$). No significant difference existed between the control group and Nar group ($P > 0.05$).

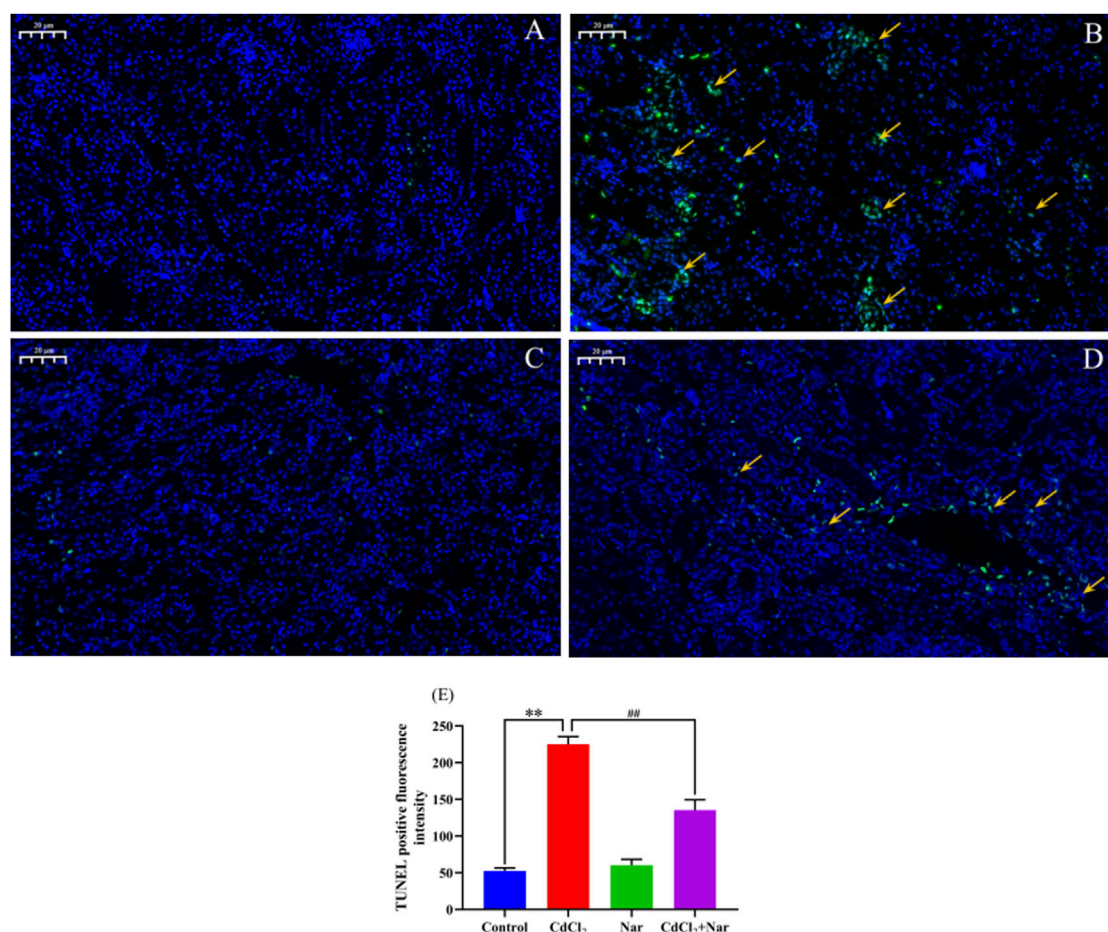


FIGURE 4

Effects of CdCl₂ and Nar on the apoptosis of chicken renal cells. (A) control group, (B) CdCl₂ group, (C) Nar group, (D) CdCl₂ + Nar group. The scale is 20 μm. Orange arrow: TUNEL apoptotic cells. (E) TUNEL positive fluorescence intensity. ** $P < 0.01$: compared with the control group. # $P < 0.05$, ## $P < 0.01$: compared with the CdCl₂ group.

3.10 Effect of CdCl₂ and Nar on autophagy-related proteins in chicken kidneys

The expression levels of autophagy-related proteins were detected to further verify the effects of CdCl₂ and Nar on autophagy (Figure 8). The results revealed that the significantly higher levels of autophagy-related proteins LC3 (78.75%), Beclin-1 (17.88%), and ATG5 (27.15%) and the significantly lower expression level of P62 (28.07%) in the CdCl₂ group than those in the control group ($P < 0.01$). The CdCl₂ + Nar co-treatment group showed significantly lower expression levels of autophagy-related proteins LC3 (38.08%), Beclin-1 (10.05%), and ATG5 (11.62%) and significantly increased expression level of P62 (27.93%) compared with those in the CdCl₂ group ($P < 0.01$). These results indicate that CdCl₂ activates renal autophagy in chickens, and Nar can alleviate CdCl₂-induced autophagy.

4 Discussion

Cd is an environmental pollutant that enters the bodies of humans and animals through the air, drinking water, and food

chain. It travels through the bloodstream to organs throughout the body and accumulates in the kidneys. Toxicological studies reveal that the relative organ weight plays an important role in evaluating toxicant effects *in vivo*. The ratio of organ weight to body weight can be calculated to reflect the toxic effects of Cd on organs (Gebrezgi et al., 2020). Studies have shown that Cd treatment can cause kidney dysfunction (Gong et al., 2022; Lian et al., 2023). BUN, UA, and Cr are commonly used indicators of kidney function. NAG is a glycosidase found in renal proximal tubules and mainly used in the detection of acute and chronic kidney diseases (Novak et al., 2023). Most researchers have found that CdCl₂ poisoning induces substantial increases in renal UA, BUN, and Cr levels (Kim et al., 2018; Salama et al., 2021; Huang et al., 2022). Śliwińska-Mossoń et al. found that long-term Cd exposure in rats resulted in increased NAG activity in the kidneys (Śliwińska-Mossoń et al., 2019). Our findings are consistent with those of previous research, which showed that Cd treatment caused a decrease in the body weight of chickens and an increase in relative weight of their kidneys. CdCl₂ treatment considerably increased the levels and activities of UA, BUN, Cr, and NAG. The Cd + Nar co-treatment group exhibited the remarkable alleviation of the weight loss and kidney injury induced

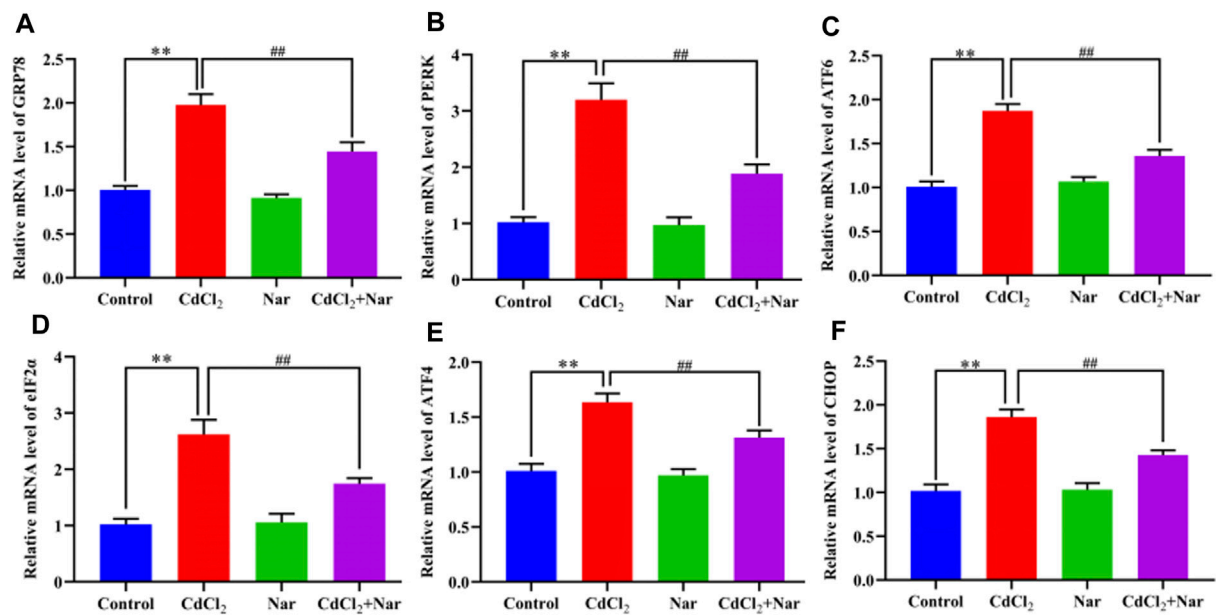


FIGURE 5 Effects of CdCl₂ and Nar on the expression levels of ER stress-related genes. (A) GRP78, (B) PERK, (C) ATF6, (D) eIF2α, (E) ATF4, (F) CHOP. Values are expressed as the mean ± SEM. ***P* < 0.01: compared with the control group. ##*P* < 0.01: compared with the CdCl₂ group.

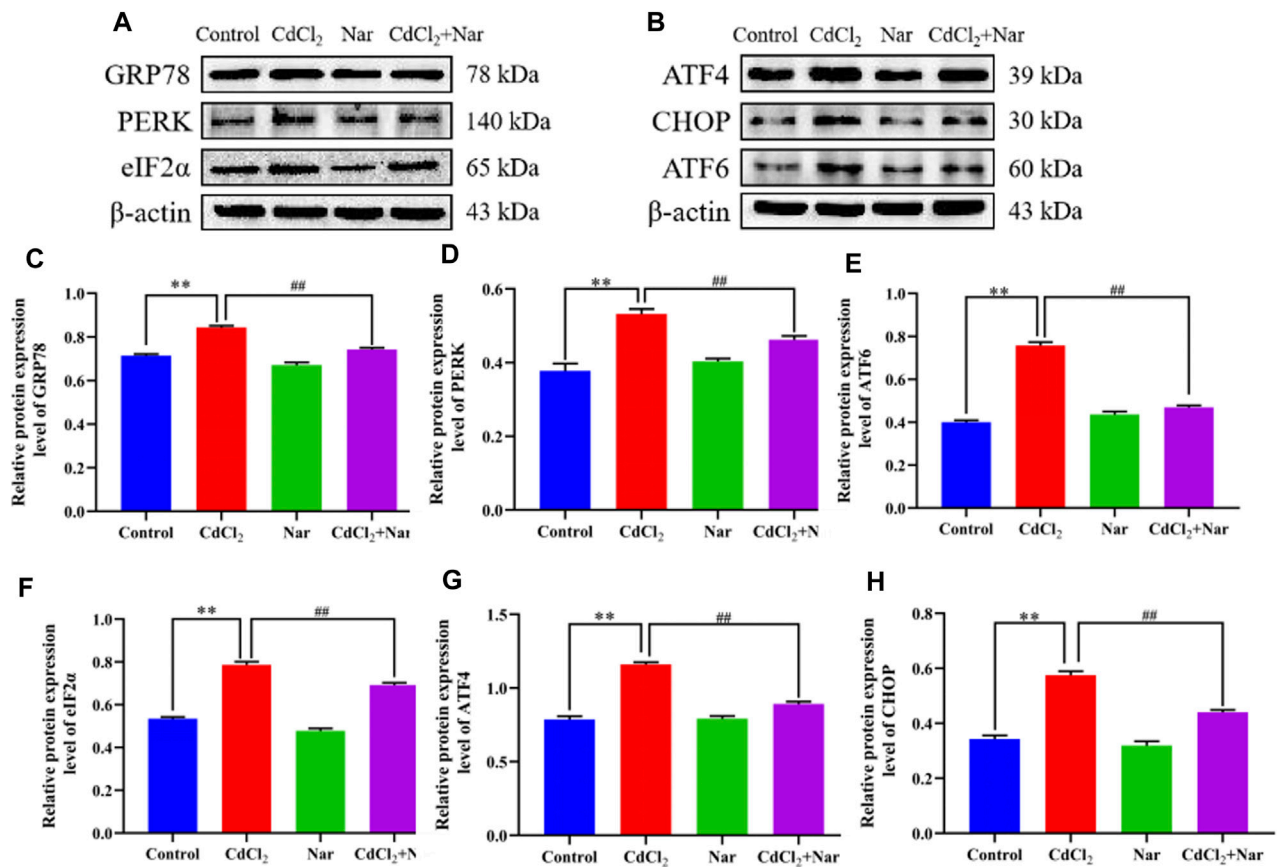


FIGURE 6 Effects of CdCl₂ and Nar on ER stress-related proteins. (A) Western blot analysis of GRP78, PERK, eIF2α, and β-actin proteins. (B) Western blot analysis of ATF4, CHOP, and ATF6 proteins. (C) GRP78, (D) PERK, (E) ATF6, (F) eIF2α, (G) ATF4, (H) CHOP. Values are expressed as the mean ± SEM. ***P* < 0.01: compared with the control group. #*P* < 0.05, ##*P* < 0.01: compared with the CdCl₂ group.

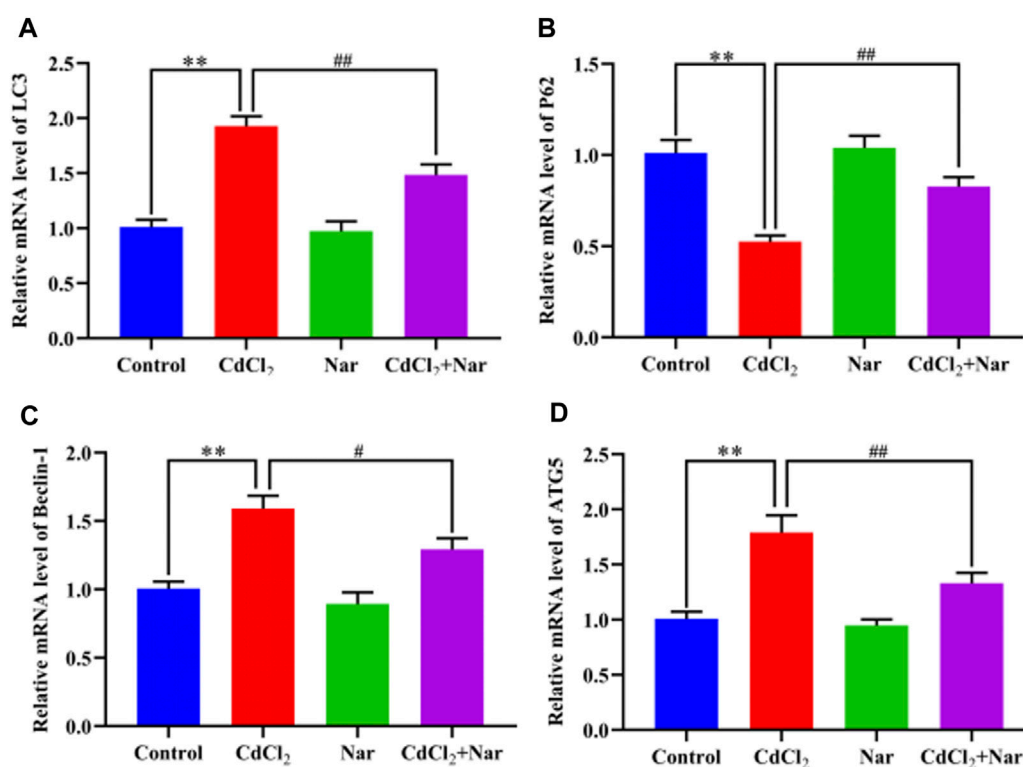


FIGURE 7
Effects of CdCl₂ and Nar on the expression levels of autophagy-related genes. (A) LC3, (B) P62, (C) Beclin-1, (D) ATG5. Values are expressed as the mean $\pm$ SEM. ** P < 0.01: compared with the control group. # P < 0.05, ## P < 0.01: compared with the CdCl₂ group.

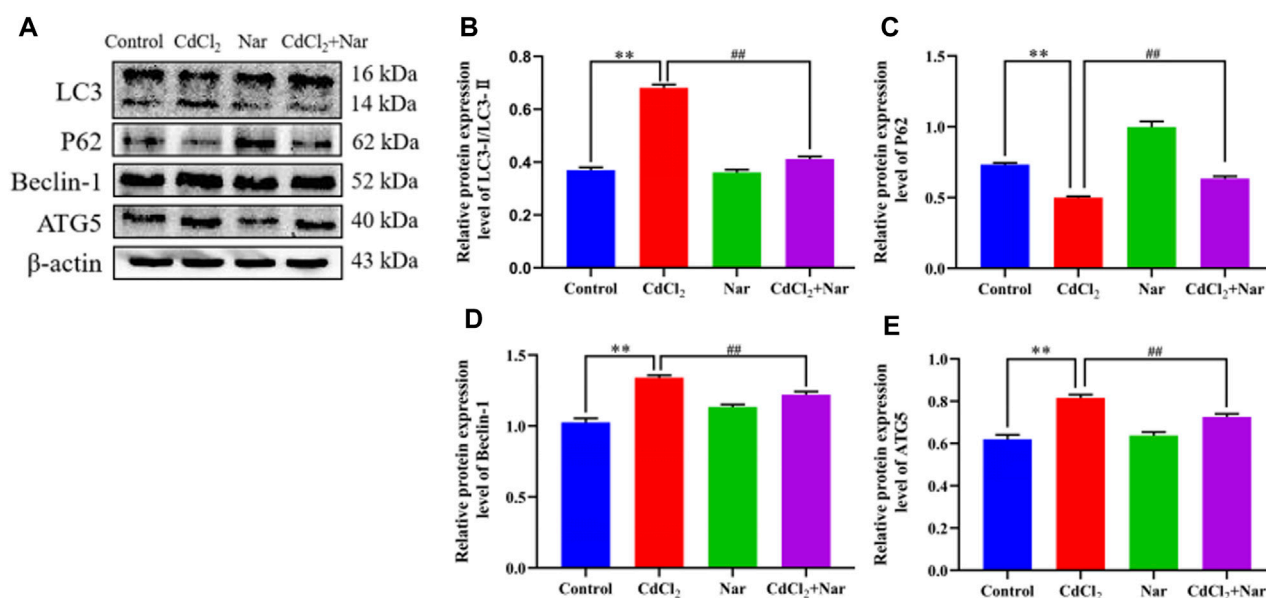


FIGURE 8
Effects of CdCl₂ and Nar on autophagy-related proteins. (A) Western blot analysis of LC3, P62, Beclin-1, ATG5, and β -actin proteins. (B) LC3-II/LC3-I, (C) P62, (D) Beclin-1, (E) ATG5. Values are expressed as the mean $\pm$ SEM. ** P < 0.01: compared with the control group. ## P < 0.01: compared with the CdCl₂ group.

by CdCl₂. These results imply that Nar imply alleviate the renal toxicity induced by CdCl₂.

OS is one of the many mechanisms underlying kidney injury caused by Cd. Studies have shown that exposure to Cd leads to the excessive production of ROS by the mitochondria and NADPH oxidase, ultimately causing OS (Yan and Allen, 2021). Sun et al. found that Cd treatment substantially elevated the MDA content in duck liver, while reducing SOD activity (Ge et al., 2019). Zhu et al. found that the levels of MDA and GSH in the kidneys of laying hens increase upon adding CdCl₂ to the diet (Zhu et al., 2019). Consistent with previous studies, the present research revealed that exposure to CdCl₂ induced OS in chicken kidneys, as proven by decreased T-AOC, SOD, and CAT levels and elevated MDA and GSH levels. Nar has a powerful antioxidant effect, and it can alleviate OS in the livers of adriamycin-poisoned rats, which led to increased SOD and CAT activities (Wali et al., 2020). Das et al. found that Nar alleviated the liver and renal toxicity of Cd in albino mice by increasing the levels of antioxidant indices, such as SOD and CAT (Das et al., 2016). In this experiment, the CdCl₂ + Nar co-treatment group exhibited significantly higher activities of T-AOC, SOD, and CAT and significantly lower MDA and GSH levels compared with the CdCl₂ group. Therefore, Nar can reduce CdCl₂-induced OS in the kidney.

In histology and pathology, H&E staining plays a key role in clearly showing the structural differences between normal and abnormal tissues, as well as in identifying pathological changes in tissues (Feldman and Wolfe, 2014). After exposure to Cd in pregnant rats, the fetal kidneys were damaged, and the renal histopathological manifestations included renal tubular injury (Jacobo-Estrada et al., 2016). Apoptosis also plays an important role in Cd-induced nephrotoxicity (Li et al., 2022). In the current work, H&E and TUNEL methods were used to investigate the impact of CdCl₂ and Nar on renal tissue injury and apoptosis in chickens. Results showed that the fluorescence intensity of the Nar group was similar to that of the control group, and the glomerular structure appeared normal based on H&E staining. The fluorescence intensity of the CdCl₂ group was significantly higher than that of the control group and Nar group. Meanwhile, kidney injury was significantly aggravated, characterized by blurred and irregular glomerular boundaries. This finding indicated that CdCl₂ may induce kidney injury and apoptosis in chickens, consistent with the findings of Li et al. (2022). Furthermore, apoptosis significantly decreased in the CdCl₂ + Nar group, and kidney injury notably improved. These results suggested that Nar alleviated CdCl₂-induced renal injury and apoptosis in chickens.

OS disrupts the ER organelles, which results in the overproduction of intracellular ROS. Such a phenomenon causes erroneous protein aggregation, which in turn induces ER stress and triggers UPR (Zhang et al., 2023). Studies have shown that the heavy metal Cd can cause ER stress *in vivo* and *in vitro* (Kitamura and Hiramatsu, 2010). He et al. (2022) observed that Cd activated liver ER stress in a mouse model of Cd treatment. Wang et al. found that Cd treatment upregulates GRP78, ATF6, IRE1, CHOP, PERK, and JNK proteins in chicken testis (M. Wang et al., 2023). Consistent with the abovementioned results, we found that ER stress-related proteins, such as GRP78, ATF6, CHOP, PERK, ATF4, and eIF2 α , can be upregulated by the addition of CdCl₂ diet to chickens. Meanwhile, the CdCl₂ + Nar co-treatment group showed reduced

expression levels of ER stress-related proteins. Nar significantly reduced the CdCl₂-induced elevations of CHOP, GRP78, ATF6, ATF4, IRE1, PERK, and XBP1s in rat liver (Ustuner et al., 2020). This result indicates that Nar alleviates CdCl₂-induced renal ER stress in chickens.

The autophagy process is crucial to maintain balance in the intracellular environment, with LC3, P62, Beclin-1, and ATG5 serving as key factors. During autophagy, LC3 is a marker of autophagy and is categorized into LC3-I and LC3-II (Kabeya et al., 2000). The LC3-II-to-LC3-I ratio is commonly used to indicate the LC3 level. P62 is a multifunctional protein involved in autophagy. It can directly bind to LC3 and ubiquitinated proteins, which are ultimately degraded in the lysosome (Bjørkøy et al., 2009). Beclin-1 and ATG5 are key regulatory proteins in autophagosome formation. Feng et al. found that the expression level of p62 considerably increased when autophagy was inhibited (Feng et al., 2017). Qiu et al. (2023) established a mouse poisoning model of Cd and PSNP and observed that combined exposure to Cd and PSNP substantially increased the expression levels of Beclin-1, ATG5, and LC3 but decreased that of P62. Consistent with the abovementioned results, we observed that Cd treatment activated autophagy in the chickens kidney by upregulating and downregulating the expression levels of LC3 and P62, respectively. Conversely, the CdCl₂ + Nar co-treatment group showed the reduced expression level of LC3 and elevated P62 level, which mitigated autophagy. Nar reduced the CdCl₂-induced elevation of LC3 in rat liver (Ustuner et al., 2020). This result provides support to our research.

5 Conclusion

In conclusion, a model of Cd poisoning in chickens was created, and Nar was added to antagonize Cd toxicity. It was found that exposure to CdCl₂ resulted in decreased body weight and impaired kidney function, as well as induced the apoptosis of renal cells, OS, ER stress, and autophagy in chicken kidneys. However, Nar can effectively combat the adverse effects of CdCl₂, relieve the CdCl₂-induced weight loss, restore kidney function, reduce OS and apoptosis, alleviate ER stress, and regulate autophagy. Furthermore, this study provides a valuable foundation for the application of Nar in CdCl₂ poisoning. However, it is still unclear how Nar regulates ER stress and autophagy induced by CdCl₂, and its mechanism needs further study.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The animal study was approved by this study was approved by the Animal Ethical and Welfare Committee of Henan University of Science and Technology (approval code: 2023048). The study was

conducted in accordance with the local legislation and institutional requirements.

Author contributions

YS: Data curation, Formal Analysis, Software, Writing—original draft. ZG: Data curation, Formal Analysis, Writing—original draft. BX: Formal Analysis, Validation, Writing—original draft. JM: Data curation, Visualization, Writing—original draft. YW: Formal Analysis, Supervision, Writing—review and editing. ZL: Conceptualization, Methodology, Validation, Writing—review and editing. JW: Methodology, Resources, Supervision, Writing—review and editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This study was supported by grants from National Natural Science Foundation of China (Nos 32273083, 31972753).

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2024.1440877/full#supplementary-material>

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RECEIVED 20 September 2024

ACCEPTED 11 October 2024

PUBLISHED 18 October 2024

CITATION

Guo Y, Yuan Y, Wang R, Bai J, Jia Y, Qiu X, Niu H,
Li L, Luo Y, Zhao B and Zhang Z (2024)
Monocrotaline-mediated autophagy via
inhibiting PI3K/AKT/mTOR pathway induces
apoptosis in rat hepatocytes.
Front. Pharmacol. 15:1499116.
doi: 10.3389/fphar.2024.1499116

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Monocrotaline-mediated autophagy via inhibiting PI3K/AKT/mTOR pathway induces apoptosis in rat hepatocytes

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Monocrotaline (MCT), a major pyrrolizidine alkaloid, is well-known for its high liver toxicity. Dysregulation of autophagy induced apoptosis can lead to various liver diseases, including those induced by chemical compounds. Therefore, we aim to explore whether autophagy might serve as a potential strategy for addressing liver apoptosis caused by MCT. In primary rat hepatocytes (PRHs), MCT significantly increased the number of autophagosomes and the expression levels of LC3II, Beclin-1, and Atg5, while it decreased the expression of p62 in a concentration-dependent manner at doses of 100, 200, 300, and 400 μ M. Western blot assays revealed MCT inhibited the phosphorylation levels of the PI3K/AKT/mTOR pathway. To elucidate the role of autophagy in mediating MCT-induced apoptosis, we further pretreated PRHs with the autophagy agonist Rapamycin and the inhibitors Bafilomycin A1 and Chloroquine, respectively, and assessed the apoptosis of PRHs induced by MCT. The results displayed that Rapamycin increased the apoptosis rate and the expression of cleaved caspase-3, whereas Bafilomycin A1 and Chloroquine reduced the apoptosis and the expression of cleaved caspase-3 in PRHs. This study confirms that autophagy enhances PRHs apoptosis induced by MCT. In summary, this study demonstrates that MCT-induced autophagy via inhibition of the PI3K/AKT/mTOR pathway can lead to apoptosis in PRHs.

KEYWORDS

monocrotaline, hepatotoxicity, autophagy, PI3K/Akt/mTOR signaling pathway, apoptosis

1 Introduction

Pyrrolizidine alkaloids (PAs) are a group of natural compounds found in approximately 3% of flowering plants (Schrenk et al., 2020). It is crucial to emphasize that more than 50% of these PAs have been proven to possess hepatotoxic effects, leading to their classification as hepatotoxic PAs (HPAs) (Prakash et al., 1999). In addition, some PAs also exhibit nephrotoxicity, teratogenicity, carcinogenicity, and genetic toxicity, with a few even

exhibiting pulmonary toxicity. Currently, it has been reported that more than 6,000 species of plants from 13 genera in natural grasslands contain PAs (Smith and Culvenor, 1981; Stegelmeier et al., 1999). These plants are predominantly found in toxic plant families such as *Compositae*, *Boraginaceae*, and *Leguminosae* (Dusemund et al., 2018). Humans are often poisoned by consuming grains or animal-derived foods contaminated with PAs, such as honey, milk, and eggs (Huybrechts and Callebaut, 2015; Mulder et al., 2018; Roncada et al., 2023). Additionally, poisoning can occur from drinking tea or herbal preparations containing PAs. Livestock are forced to consume plants containing PAs during seasons when edible forage is severely scarce, leading to chronic poisoning, and even death, which poses a significant threat to the economic sustainability of grassland livestock farming (Guo et al., 2020).

Monocrotaline (MCT) belongs to the PAs and is mainly derived from plants in the *Crotalaria* genus (Roeder, 1995). Its structure is a macrocyclic diester consisting of 11 members and lacking an α , β -unsaturated double bond. MCT exhibits low toxicity, but after metabolism in the liver, it forms intermediate metabolites that can rapidly bind to nucleophilic substances, such as proteins containing thiol (-SH), hydroxyl (-OH), and amino (-NH) groups, as well as DNA and RNA, leading to liver damage (Gong et al., 2023). However, the mechanisms of MCT-induced liver injury remain controversial. Our previous study have demonstrated that endoplasmic reticulum stress is a key factor contributing to MCT-induced apoptosis in hepatocytes (Guo et al., 2021). Additionally, research has shown that Kupffer cells play a crucial role in MCT-induced liver injury by producing TNF- α (Cao et al., 2022). Therefore, further investigation is required to fully understand the mechanisms underlying MCT-induced hepatocyte damage.

MCT can induce hepatocyte death (Copple et al., 2004). Cell death can occur through programmed cell death (PCD), including mainly apoptosis, autophagy, necroptosis and ferroptosis. Apoptosis is characterized by DNA fragmentation, chromatin condensation, membrane blebbing and cell shrinkage. During apoptosis, proteins encoded by the Bcl-2 gene family, such as Bcl-2, Bax, along with caspases like caspase-3 and caspase-9, serve as crucial mediators and key executors of the process. Previous studies have found that MCT-induced endoplasmic reticulum stress is one of the causes of hepatocyte apoptosis (Guo et al., 2021). Autophagy, another form of PCD, utilizes autophagosomes to break down unnecessary or damaged organelles, as well as cells exposed to toxins. This process is dynamically regulated by several proteins that coordinate the formation of the autophagic membrane, the engulfment of autophagosomes, and their subsequent fusion with lysosomes, involving key players such as LC3 and p62 (Gao et al., 2020; Allaire et al., 2019; Ichimiya et al., 2020). Nervosine VII, a compound belonging to PAs and extracted from *Liparis nervosa*, has been found to induce autophagy in human colorectal cancer cells (Huang et al., 2020). Nevertheless, the mechanism by which MCT triggers autophagy in the liver remains elusive. In this paper, we observed the effect of MCT on autophagy by examining the PI3K/AKT/mTOR pathway. Additionally, by introducing autophagy inhibitors and agonists, we further investigated the impact of MCT on cell apoptosis, thereby investigating the role of autophagy in MCT-induced hepatocyte apoptosis.

2 Materials and methods

2.1 Reagent

Monocrotaline (purity >98%, CAS No. 315-22-0) was purchased from Sigma Aldrich (United States, Catalog No. C2401) and the stock concentration of 50 mM MCT was prepared by dissolving in 1 mol/L HCl and balanced the pH to 7.0–7.4 by adding 5 mmol NaOH. DMEM medium (Gibco, United States, Catalog No. 12800017) containing 10% FBS (Zeta Life, United States, Catalog No. Z7181FBS) was used for cell culture. Bafilomycin A1 A1 (Catalog No. HY-100558), Rapamycin (Catalog No. HY-10219) and Chloroquine (Catalog No. HY-17589A) were obtained from MCE, United States. Ad-GFP-LC3B was purchased from Beyotime (Shanghai, China).

2.2 Cell culture and drug treatment

Primary rat hepatocytes (PRH) were prepared as previously described (Guo et al., 2021) and male Sprague–Dawley rat was obtained from Cheng Du Dossy Biological Technology Co., Ltd. (Sichuan, China). PRH were seeded at a density of 1×10^6 cells/mL in 6-well plates and maintained in a 37°C incubator with 5% CO₂. After 24 h cultured, PRH were treated with different concentrations of MCT (0, 100, 200, 300 and 400 μ M) for different time (0, 6, 12, 24 and 36 h), or pretreat the cells with Rapamycin (50 nM), Chloroquine (15 μ M), or Bafilomycin A1 (10 nM) for 4 h. After removing the medium, replace it with fresh medium containing Rapamycin (50 nM), Chloroquine (15 μ M), Bafilomycin A1 (10 nM), Rapamycin + MCT, Chloroquine + MCT, or Bafilomycin A1 + MCT. In the control group, add an equal volume of fresh medium. Incubate the cells at 37°C for an additional 36 h. Then, the cells were collected for subsequent biological analysis.

2.3 Western blot analysis

Hepatocytes underwent lysis in an ice-cooled radioimmunoprecipitation (RIPA) buffer fortified with PMSF (R0010, Solarbio, China). Subsequently, the lysates were subjected to centrifugation at 4°C and 12,000 g for 10 min to isolate the supernatant. The protein content within the supernatant was quantified utilizing the BCA assay (PC0020; Solarbio, China). Each sample was then fractionated by SDS-PAGE (10%–15% gel) at 100 V for 1.5 h, followed by transfer onto a polyvinylidene difluoride (PVDF) membrane (Catalog No. BSP0161, PALL, United States). Prior to antibody probing, the membranes were blocked with 5% non-fat milk dissolved in TBS-T for 2 h at ambient temperature. Subsequently, the membranes were incubated overnight at 4°C with the primary antibodies of interest, diluted in an appropriate solution. LC3B (1:1,000, Catalog No.: ab192890), Beclin-1 (1:1,000, Catalog No. ab302670), cleaved capase-3 (1:1,000, Catalog No. ab214430) and Atg5 (1:1,000, Catalog No. ab108327) were purchased from Abcam (United States). p62 (1:1,000, Catalog No. 39749), PI3K (1:1,000, Catalog No. 4249), p-PI3K (1:1,000, Catalog No. 17366), AKT (1:1,000, Catalog No. 9272),

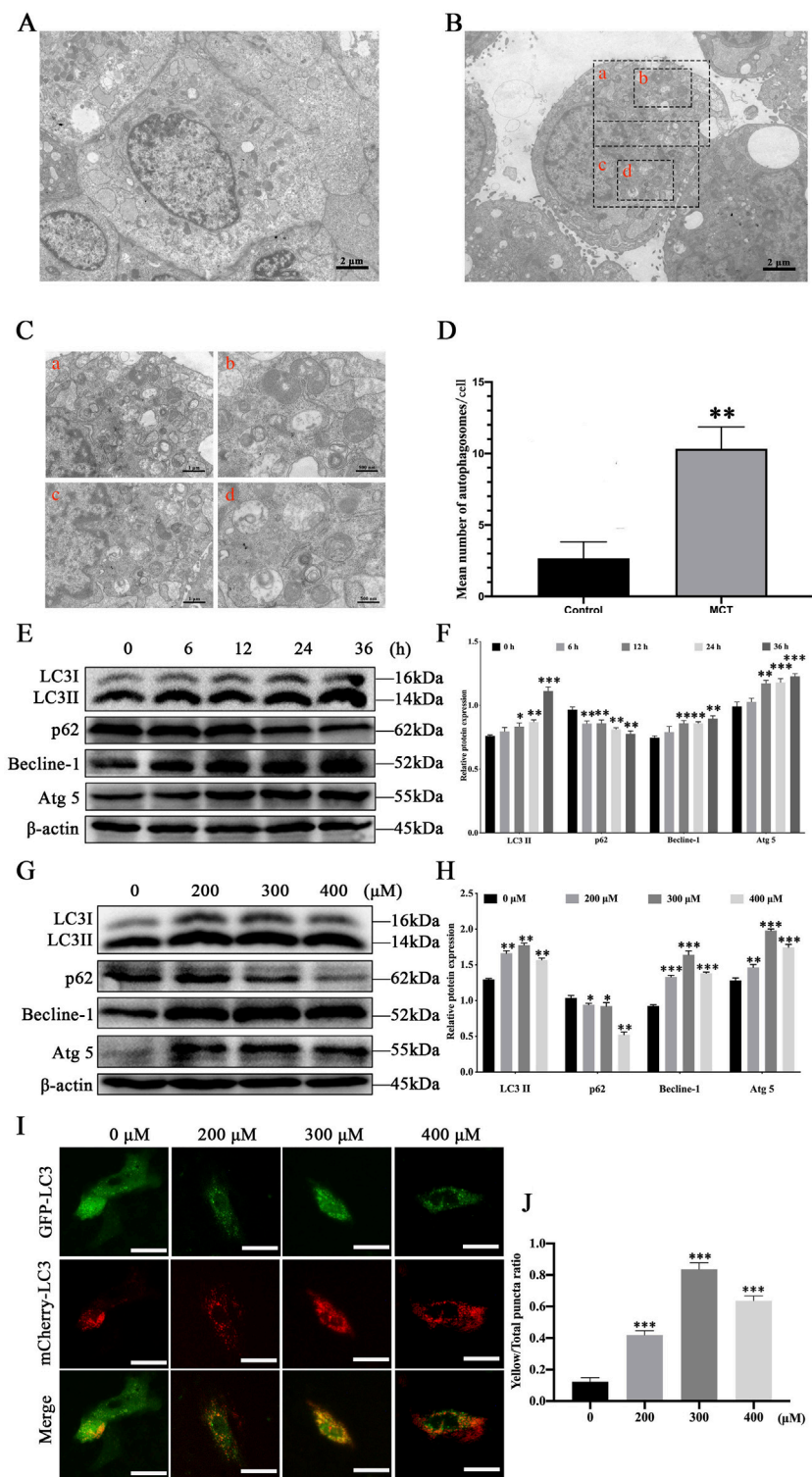


FIGURE 1
MCT induces autophagy in PRHs. **(A)** The ultrastructure of PRHs not exposed to MCT. **(B)** MCT increases the formation of autophagosome-like vesicles. **(C)** Magnified views of the autophagosome-like vesicles are enclosed by four black square frames **(A–C)** in part **(B)**. Scale bars, 1 μ m **(A, C)** and 500 nm **(B, D)**. **(D)** Quantification of the number of autophagosome-like vesicles per cell profile in cells with/without MCT. The average number of the vesicles in each cell was obtained from at least 10 cells per experimental condition. **(E)** Detection of autophagy-related protein expression induced by 300 μ M MCT over different time (0, 6, 12, 24 and 36 h), including LC3II, Beclin-1, p62, Atg5 and β -actin (loading control) by Western blot. **(F)** Quantitative analysis of protein levels compared to β -actin protein levels was determined by densitometry in **(E)**. **(G)** Detection autophagy-related protein expression induced by different concentrations (0, 200, 300 and 400 μ M) of MCT after 36 h, including LC3II, Beclin-1, p62, Atg5 and β -actin (loading control) *(Continued)*

FIGURE 1 (Continued)

control) by Western blot. (H) Quantitative analysis of protein levels compared to β -actin protein levels was determined by densitometry in (G). (I) MCT promotes the formation of autophagic fluorescent puncta in PRHs. Scale bars, 100 μ m. (J) Quantified the yellow fluorescent puncta ratio of autophagy in PRHs. Data are presented as mean $\pm$ SD of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 compared to control.

p-AKT (1:1,000, Catalog No. 4060), mTOR (1:1,000, Catalog No. 2972) p-mTOR (1:1,000, Catalog No. 2971) and β -actin (1:2000, Catalog No. 4970). The membranes were incubated with a goat anti-rabbit IgG-HRP secondary antibody (1:5,000, Beyotime Institute of Biotechnology, China).

2.4 Transmission electron microscopy (TEM)

PRH were seeded in 6-well plates and incubated at 37°C for 24 h. The control group was cultured under standard conditions, while the treatment group received 300 μ M MCT. After 36 h, cells were harvested, washed three times with PBS, and fixed in 4% glutaraldehyde. Postfixation was carried out in 1% OsO₄ in 0.1 M cacodylate buffer containing 0.1% CaCl₂ for 2 h at 4°C. Samples were stained with 1% Millipore-filtered uranyl acetate, dehydrated with a graded ethanol series, infiltrated, and embedded. Following resin polymerization at 60°C for 48 h, ultrathin sections were cut using an ultramicrotome, stained with 4% uranyl acetate and lead citrate, and imaged with a transmission electron microscope (FEI Tecnai G2 Spirit Bio TWIN, United States).

2.5 Fluorescence microscopy

PRH were seeded into 35 mm culture dishes for 4 h, the medium was replaced. Fresh medium containing 30 MOI Ad-mCherry-GFP-LC3B was added, and the cells were incubated at 37°C for 24 h. After removing the medium containing Ad-mCherry-GFP-LC3B, fresh medium or medium containing different concentrations of MCT (200, 300, and 400 μ M) was added, and incubation continued. The distribution of GFP-LC3B green fluorescent puncta and mCherry-LC3B red fluorescent puncta was observed under a fluorescence microscope (OLYMPUS-IX71, Japan).

2.6 Apoptosis detection by annexin V/PI double staining

The rate of cell apoptosis was assessed using the Annexin V-FITC Apoptosis Detection Kit (Dojindo, Catalog No.: AD10, Japan) following the manufacturer's protocol. In summary, PRH were collected and washed twice with cold PBS. The cells were then resuspended in binding buffer and incubated with 5 μ L of annexin V-FITC and 5 μ L of PI for 15 min at room temperature in the dark. Apoptosis was analyzed using a BD FACSARIA™ III flow cytometer (BD, United States) within 1 h, and the data were processed using Treestar FlowJo software (United States).

2.7 Statistical analysis

All results are expressed as the mean $\pm$ SD (with vertical error bars) from triplicate experiments. Differences between groups were assessed using One-way ANOVA, performed with GraphPad Prism version 10.0 software (San Diego, CA, United States). p < 0.05 was indicated a statistically difference and p < 0.01 was indicated significantly statistically difference.

3 Result

3.1 MCT increases the level of autophagic markers in PRHs

To determine whether MCT induces autophagosome formation, PRHs were treated with 300 μ M MCT for 36 h, followed by ultrastructural analysis. Compared to control group (Figure 1A), the number of vesicle-like structures resembling single- or double-membrane autophagosomes significantly increased in MCT-treated cells (Figure 1B). Additionally, regional magnification of the cells revealed that these vesicles contained numerous contents (Figure 1C). In comparison, such vesicle-like structures were rarely observed in control group (Figure 1D). These results indicate that MCT can induce autophagy in PRHs and promote the formation of autophagosome structures.

To further investigate autophagy induced by MCT in PRHs, autophagy-related marker proteins were analyzed using Western blotting. After treatment with 300 μ M MCT for 0, 6, 12, 24 and 36 h, the expression levels of autophagy markers LC3II, Beclin-1, and Atg5 in hepatocytes significantly increased over time, while the expression of p62 protein markedly decreased (Figures 1E, F). When cells were treated with different concentrations of MCT (0, 200, 300 and 400 μ M) for 36 h, the expression levels of LC3II, Beclin-1, and Atg5 were significantly elevated compared to the control group (Figures 1G, H). Autophagic fluorescent puncta were observed using a fluorescence microscope. As shown in Figures 1I, J, after 36 h of MCT treatment in PRHs, which is infected with the Ad-mCherry-GFP-LC3B, the control group displayed a uniform distribution of GFP-LC3B green fluorescence with only a few bright green fluorescent puncta visible. The mCherry-LC3B red fluorescent puncta were relatively scarce in the cells, resulting in a low number of yellow puncta when they were merged. In contrast, the MCT-treated group showed a significant increase in the number of green fluorescent puncta, along with a marked increase in mCherry-LC3B red fluorescent puncta. Consequently, the proportion of yellow fluorescent puncta in MCT-treated hepatocytes was significantly higher. These results indicate that MCT promotes autophagy in PRHs.

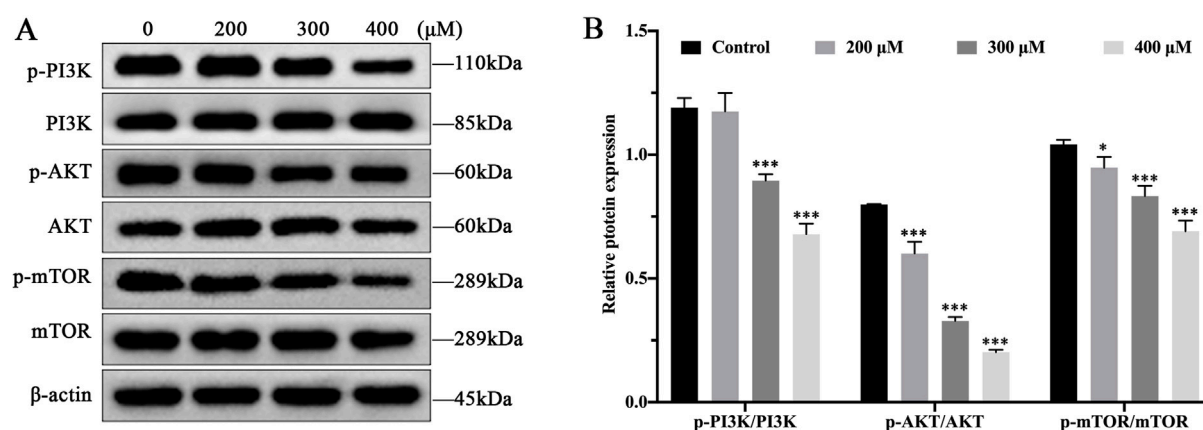


FIGURE 2
MCT inhibits the phosphorylation levels of PI3K/AKT/mTOR pathway in PRHs. **(A)** Detection of autophagy-related protein expression induced by different concentrations (0, 200, 300 and 400 μ M) of MCT after 36 h, including PI3K, p-PI3K, AKT, p-AKT, mTOR, p-mTOR and β -actin (loading control) by Western blot. **(B)** The phosphorylation levels of proteins were quantitatively analyzed relative to their corresponding non-phosphorylated proteins using densitometry in **(A)**. Data are presented as mean $\pm$ SD of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 compared to control.

3.2 MCT inhibits the expression of the PI3K/AKT/mTOR pathway in PRHs

To explore the effect of MCT on PI3K/AKT/mTOR pathway, Western blot was used to assess the expression level of p-PI3K, p-AKT, and p-mTOR in PRHs, treated with different concentrations of MCT (0, 200, 300 and 400 μ M). As shown in Figure 2, compared to the control group, the expression of p-PI3K, p-AKT and p-mTOR proteins showed a significant reduction with increasing concentrations of MCT. These results suggest that MCT can inhibit the phosphorylation of proteins in the PI3K/AKT/mTOR pathway.

3.3 Activation of autophagy enhances MCT-induced apoptosis in PRHs

PRHs were divided into four groups (control, Rapamycin (50 nM)-treated group, MCT (300 μ M)-treated group and Rapamycin (50 nM) + MCT (300 μ M)-treated group). The expression of autophagy-related proteins was assessed. As shown in Figures 3A, B, compared to the control group, there was no significant change in the expression levels of LC3II, p62, Beclin-1, and Atg5 proteins in the Rapamycin-treated group. However, in the Rapamycin + MCT-treated group, the expression of LC3II, Beclin-1, and Atg5 proteins was significantly increased, while the expression of p62 was significantly decreased compared to the MCT-treated group. These results indicate that Rapamycin can promote MCT-induced autophagy in PRHs.

We used flow cytometry to confirm the role of Rapamycin in MCT-induced apoptosis in PRHs. As shown in Figures 3C, D, Rapamycin pretreatment did not significantly affect apoptosis. However, the apoptosis rate was significantly increased in the Rapamycin + MCT-treated group. Additionally, Western blot was performed to detect the expression of cleaved caspase-3. As shown in Figures 3E, F, Rapamycin pretreatment did not significantly alter

cleaved caspase-3 expression compared to the control group. In contrast, cleaved caspase-3 expression was significantly elevated in the Rapamycin + MCT-treated group compared to the MCT-treated group. These results indicate that Rapamycin can promote MCT-induced apoptosis in PRHs.

3.4 Inhibition of autophagy attenuates MCT-induced apoptosis in PRHs

To further investigate the role of autophagy in MCT-induced apoptosis, PRHs were divided into six groups (control, Chloroquine (15 μ M)-treated group, Bafilomycin A1 (10 nM)-treated group, MCT (300 μ M)-treated group, Chloroquine (15 μ M) + MCT (300 μ M)-treated group and Bafilomycin A1 (10 nM) + MCT (300 μ M)-treated group). The expression of autophagy-related proteins was assessed. As illustrated in Figures 4A, B, treatment with MCT significantly upregulated the expression of LC3II, Beclin-1, and Atg5, while the levels of p62 were markedly reduced compared to the control group. In contrast, co-treatment with Chloroquine and MCT significantly decreased the expression of LC3II, Beclin-1, and Atg5, and increased p62 levels compared to the MCT group. Similarly, Bafilomycin A1 combined with MCT treatment led to a significant reduction in LC3II, Beclin-1, and Atg5 expression, accompanied by an increase in p62 levels. These findings suggest that Chloroquine and Bafilomycin A1 effectively inhibit MCT-induced autophagy in PRHs.

Flow cytometry was employed to assess the impact of these autophagy inhibitors on apoptosis. As shown in Figures 4C, D, Chloroquine and Bafilomycin A1 pre-treatment did not significantly affect apoptosis rates compared to the control group. However, when compared to the MCT-treated group, the combination of Chloroquine or Bafilomycin A1 with MCT led to a marked reduction in apoptosis rates. Furthermore, Western blot analysis of the apoptotic marker cleaved caspase-3, as shown in Figures 4E, F, revealed no significant changes in its expression in cells pre-treated

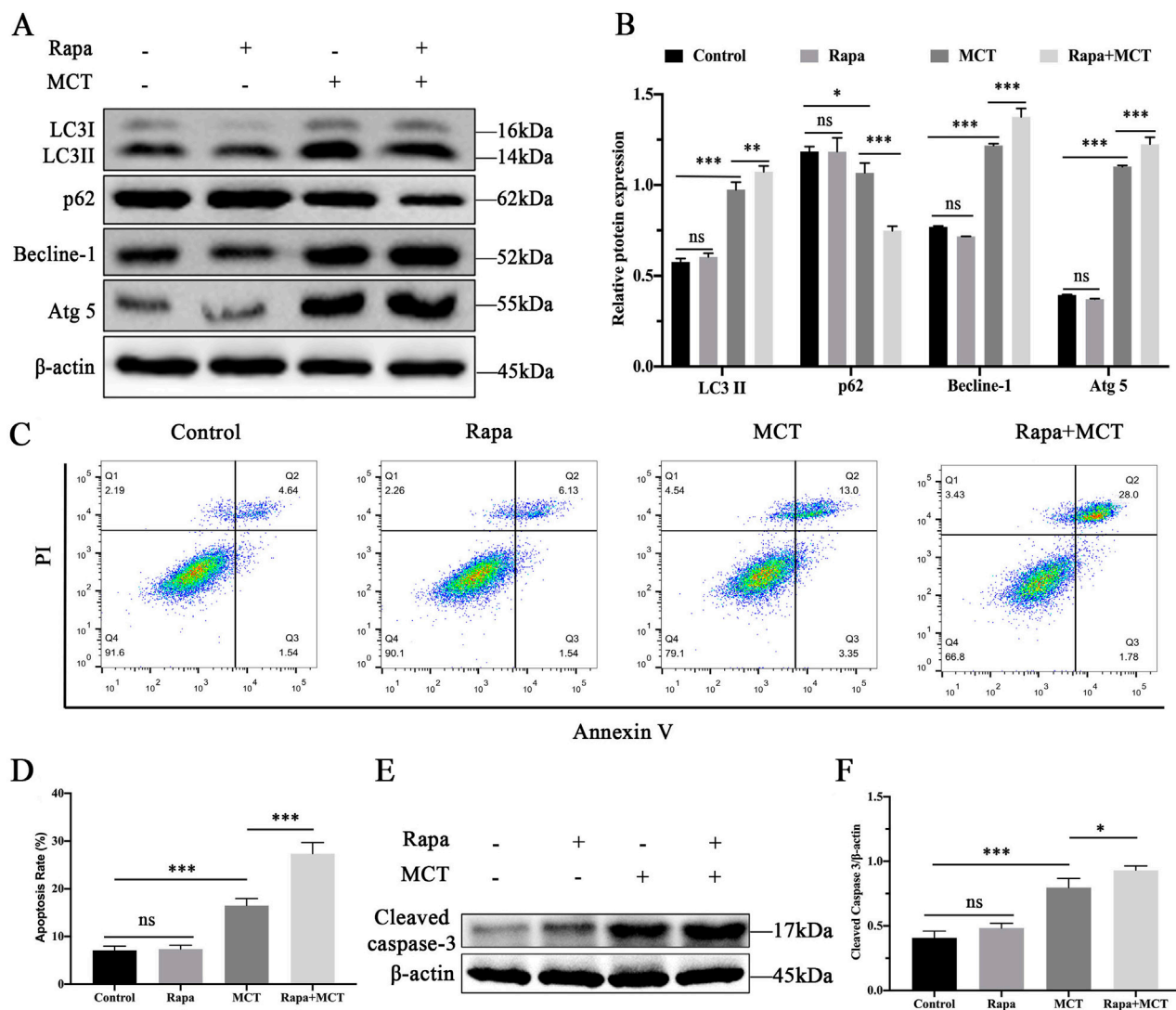


FIGURE 3

Activation of autophagy enhances MCT-induced apoptosis in PRHs. PRHs were divided into four groups (control, Rapamycin (50 nM)-treated group, MCT (300 μM)-treated group, and Rapamycin (50 nM) + MCT (300 μM)-treated group) for 36 h. (A) Detection of autophagy-related protein expression (LC3II, Beclin-1, p62, Atg5 and β-actin (loading control)) by Western blot. (B) Quantitative analysis of protein levels compared to β-actin protein levels was determined by densitometry in (A). (C) The percentages of apoptosis cells were measured by flow cytometry. Q1 quadrant stands for cell death induced by mechanical damage or necrotic cells, Q2 quadrant stands for late apoptosis cells, Q3 quadrant stands for early apoptosis cells, Q4 quadrant stands for normal cells. The sum of cell apoptosis included the early and late apoptosis cells. (D) The results of quantitative analyses of apoptosis rate. (E) Detection of cleaved caspase-3 by Western blot. (F) Quantitative analysis of protein levels compared to β-actin protein levels was determined by densitometry in (C). Data are presented as mean ± SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control.

with Chloroquine or Bafilomycin A1 alone. In contrast, co-treatment with MCT significantly reduced cleaved caspase-3 levels in the presence of either inhibitor. These findings suggest that inhibition of autophagy attenuates MCT-induced apoptosis in PRHs.

4 Discussion

Monocrotaline (MCT), a type of pyrrolizidine alkaloid (PA), is known to cause liver damage. Our previous study has demonstrated that MCT contributes to the development of apoptosis in rat hepatocytes (Guo et al., 2020). Autophagy and apoptosis are

interconnected processes involved in PCD, and study has shown that PAs can cause autophagy (Huang et al., 2020). The interaction between autophagy and apoptosis may provide insights into the impact of MCT on hepatocyte function under pathological conditions. Therefore, this study investigates the role of autophagy in MCT-induced apoptosis of PRHs.

Autophagy is fundamental in maintaining cellular energy and metabolic balance. Its role in regulating cell survival varies depending on changes and stimuli in both the intracellular and extracellular environments (Levy et al., 2017). Under certain conditions, basal autophagy was reported to provide the necessary energy and resources for cell survival and effectively prevent chronic damage to tissues and organs, thereby

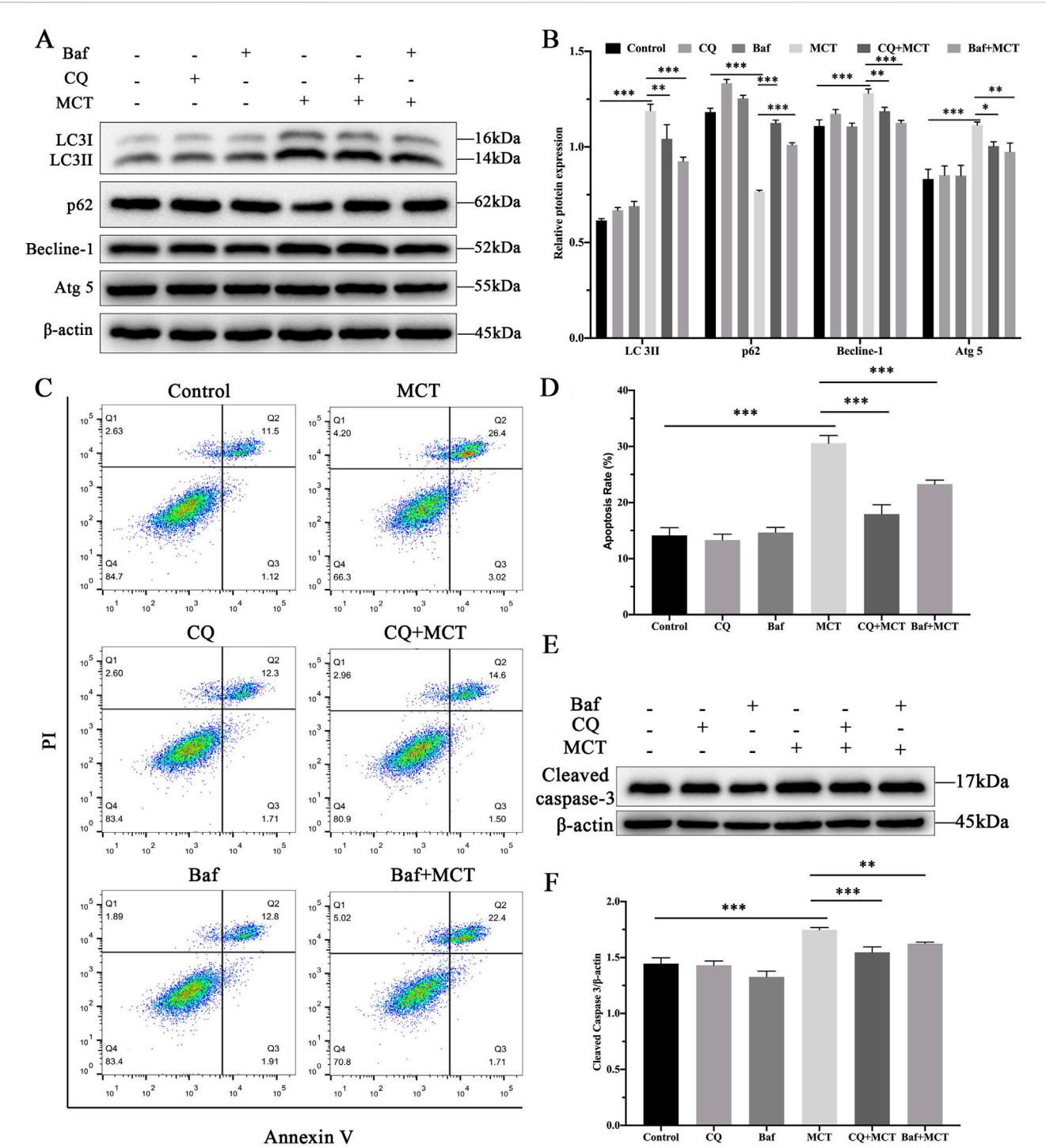


FIGURE 4
Inhibition of autophagy attenuates MCT-induced apoptosis in PRHs. PRHs were divided into six groups (control, Chloroquine (15 μ M)-treated group, Bafilomycin A1 (10 nM)-treated group, MCT (300 μ M)-treated group, Chloroquine (15 μ M) + MCT (300 μ M)-treated group, and Bafilomycin A1 (10 nM) + MCT (300 μ M)-treated group) and incubated for 36 h. **(A)** Detection of autophagy-related protein expression (LC3II, Beclin-1, p62, Atg5 and β -actin (loading control)) by Western blot. **(B)** Quantitative analysis of protein levels compared to β -actin protein levels was determined by densitometry in **(A)**. **(C)** The percentages of apoptosis cells were measured by flow cytometry. Q1 quadrant stands for cell death induced by mechanical damage or necrotic cells, Q2 quadrant stands for late apoptosis cells, Q3 quadrant stands for early apoptosis cells, Q4 quadrant stands for normal cells. The sum of cell apoptosis included the early and late apoptosis cells. **(D)** The results of quantitative analyses of apoptosis rate. **(E)** Detection of cleaved caspase-3 by Western blot. **(F)** Quantitative analysis of protein levels compared to β -actin protein levels was determined by densitometry in **(C)**. Data are presented as mean $\pm$ SD of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 compared to control.

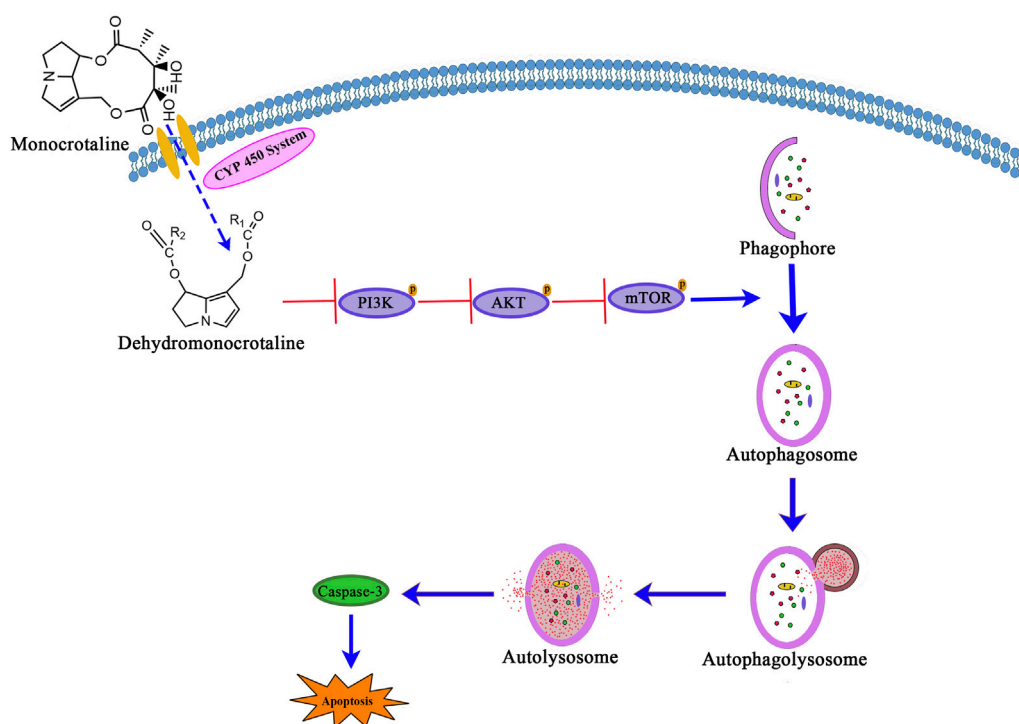


FIGURE 5
The signaling pathway involved in MCT-induced autophagy via PI3K/AKT/mTOR pathway inhibition leads to apoptosis in PRHs.

supporting cell viability (Debnath et al., 2023). Conversely, when sustained by environmental stimuli, autophagy can trigger apoptotic pathways, leading to excessive cell death and consequent damage to tissues and organs (Nassour et al., 2019). Our study found that MCT induces autophagy in PRHs, as demonstrated by a marked increase in the number of autophagosomes. Previous research has demonstrated that LC3II expression levels correlate with the number of autophagosomes, while Becline-1 is a key protein involved in autophagosome formation. Atg5 is also a critical component of the autophagosome assembly complex, and the degradation of p62 serves as a marker of completed autophagy (Kan et al., 2022). Our study found a significant increase in LC3II, Becline-1 and Atg5 levels, accompanied by a notable decrease in p62 expression. With fluorescence microscopy, we found that at a concentration of 300 μ M MCT, the number of yellow puncta peaked, suggesting enhanced autophagy. However, at a concentration of 400 μ M MCT, while the number of yellow puncta decreased, there was a notable increase in red puncta, indicating lysosomal-autophagosome fusion and enhanced autophagic flux. These results indicate that MCT not only promotes the formation of autophagosomes in PRHs but also enhances the level of autophagic flux.

Autophagy is essential for maintaining energy and nutrient balance in the liver and plays a significant role in regulating its pathophysiological processes (Mizushima and Komatsu, 2011). Recent evidence increasingly suggests that the PI3K/AKT/mTOR pathway is a critical regulator of autophagy and is involved in the initiation and progression of various pathological disorders (Yu et al., 2024). In this study, we showed that MCT inhibits the phosphorylation levels of PI3K/AKT/mTOR pathway. It is also

one of the primary mechanisms by which MCT regulates autophagy in PRHs.

Numerous studies have established that autophagy is a vital pathway for promoting cell survival under environmental stress, but it can also lead to apoptosis (Nilsson et al., 2013). To elucidate the role of autophagy in MCT-induced apoptosis of PRHs, we examined the alterations in autophagy and apoptosis-associated proteins to better understand the interplay between these two processes. This approach allowed us to gain a more comprehensive understanding of how autophagy influences cell fate under MCT treatment. Rapamycin, a known autophagy activator, facilitates the induction of autophagy through the inhibition of mTOR protein expression (Zhang et al., 2018). Following treatment with the autophagy activator, there was no significant difference observed compared to the control group. However, in the combined treatment with MCT and Rapamycin, a notable increase in the expression levels of LC3II, Becline-1, and Atg5 proteins was observed, along with a significant decrease in p62 protein levels. This suggests an enhancement of autophagic degradation. Furthermore, apoptosis analysis demonstrated that co-treatment with MCT and Rapamycin led to a higher rate of apoptosis in PRHs, as indicated by a significant increase in cleaved caspase-3 protein expression compared to the MCT-treated group. This effect may result from the intensified autophagic flux, which augments cellular degradative processes, disrupts cellular homeostasis, and ultimately triggers cell death.

In this study, Chloroquine and Bafilomycin A1 were utilized as autophagy inhibitors. Chloroquine primarily reduces lysosomal acidity, thereby impairing autophagosome degradation (Deretic, 2021). In contrast, Bafilomycin A1 inhibits the fusion of lysosomes with autophagosomes (Yoshimori et al., 1991). The

results showed that the combined treatment of MCT with either Chloroquine or Bafilomycin A1 significantly reduced the expression levels of LC3II, Beclin-1, and Atg5, while notably increasing p62 expression compared to the MCT-treated group, indicating a suppression of autophagic degradation. When comparing the apoptosis rates between the MCT-treated group and those treated with a combination of MCT and Chloroquine or MCT and Bafilomycin A1, it was observed that the co-treatment groups exhibited a significant reduction in apoptosis rates, accompanied by a marked decrease in the expression of the apoptosis-related protein cleaved caspase-3. This observation may be due to the blockade of autophagic flux, which mitigates the intense degradation associated with autophagy, thereby maintaining cellular homeostasis and reducing damage to normal cells.

5 Conclusion

In conclusion, our study demonstrates that MCT induces autophagy in PRHs by inhibiting the PI3K/AKT/mTOR signaling pathway (Figure 5). Additionally, autophagy plays a significant role in the hepatotoxicity induced by MCT. Persistent autophagy can lead to excessive cellular metabolism and impair normal hepatic function, leading to PRHs apoptosis.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by the Animal Welfare Committee of Yangling Vocational and Technical College for studies involving animals (permit number: DG 20230106). The study was conducted in accordance with the local legislation and institutional requirements.

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Author contributions

YG: Conceptualization, Methodology, Writing–original draft. YY: Data curation, Methodology, Writing–original draft. RW: Software, Writing–review and editing. JB: Data curation, Writing–review and editing. YJ: Supervision, Writing–review and editing. XQ: Project administration, Writing–review and editing. HN: Writing–review and editing. LL: Formal Analysis, Writing–review and editing. YL: Investigation, Writing–review and editing. BZ: Validation, Writing–review and editing. ZZ: Funding acquisition, Validation, Writing–review and editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was supported by the grants from the Special Scientific research project of Shaanxi Provincial Education Department (No. 22JK0629), Studio Construction Project of “PhD + vocational students” in Yangling vocational and technical college (No. BG2022004), Shaanxi Agricultural Science and Technology Innovation Drive Project (NYKJ-2022-YL52).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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OPEN ACCESS

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RECEIVED 14 August 2024

ACCEPTED 23 October 2024

PUBLISHED 05 November 2024

CITATION

Tang W, Pan Y, Zhu C, Lou D, Peng F, Shi Q and
Xiao Y (2024) DDIT4/mTOR signaling pathway
mediates cantharidin-induced hepatotoxicity
and cellular damage.
Front. Pharmacol. 15:1480512.
doi: 10.3389/fphar.2024.1480512

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DDIT4/mTOR signaling pathway mediates cantharidin-induced hepatotoxicity and cellular damage

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Background: Cantharidin (CTD) extracted from the traditional Chinese medicine *Mylabris* has significant therapeutic effects on various tumors. However, the high toxicity of CTD can cause serious liver damage, although the related molecular mechanisms remain unclear.

Methods: In this study, we established models of CTD-induced liver and L-O2 cell damage in mice *in vivo* and *in vitro*. Subsequently, liver function indicators were detected in mouse serum, while liver tissues were subjected to pathological and transmission electron microscopy observations. L-O2 cell activity was investigated using the CCK-8 assay, and the mRNA and protein expression of DNA damage-induced transcription factor 4 (DDIT4) in liver tissue and L-O2 cells was detected using qPCR, immunohistochemistry, and western blotting. Western blotting was also used to detect the expression levels of autophagy- and apoptosis-related proteins in liver tissue and L-O2 cells. After RNAi interference with DDIT4, Rap, and 3-MA treatment, autophagy and apoptosis of L-O2 cells were detected using western blotting, flow cytometry, transmission electron microscopy, and confocal microscopy.

Results: Following CTD exposure, the mouse liver showed significant pathological damage and an increase in autophagic lysosomes, while the vitality of L-O2 cells showed a significant decrease. CTD led to a significant increase in the mRNA and protein levels of DDIT4 in both liver tissue and L-O2 cells, as well as a significant increase in LC3-II, Beclin1, and Bax, whereas p-mTOR and Bcl-2 were significantly decreased. Following DDIT4 interference and 3-MA treatment, the levels of autophagy and apoptosis induced by CTD in L-O2 cells were reduced. After Rap treatment, both autophagy and apoptosis of CTD-induced L-O2 cells were significantly enhanced.

Conclusion: The molecular mechanism of CTD-induced toxicity in mouse liver and L-O2 cells is mainly through DDIT4/mTOR signaling pathway activation, leading to an increase in autophagy and apoptosis levels.

KEYWORDS

cantharidin, liver injury, DDIT4, mTOR signaling pathway, autophagy, apoptosis

1 Introduction

Mylabris, a traditional Chinese medicine, is the earliest discovered anti-tumor insect drug and has a history of more than 2000 years. *Mylabris* is the dried body of *Mylabris phalerata* Pallas or *Mylabris scichorii* Linnaeus and was first recorded in the “Shen Nong’s Herbal Classic” (Wang, 1989; Wang G. et al., 2018). Cantharidin (CTD) is the main active ingredient extracted from *Mylabris*. Modern pharmacological research has demonstrated that CTD has a strong killing effect on liver, lung, gastric, bladder, breast, and pancreatic cancers, as well as other stubborn tumors (Naz et al., 2020; Ren and Kinghorn, 2021). Several *Mylabris*-/CTD-based drug formulations are available in the Chinese market, including Aidi injection, compound *Mylabris* capsules, and disodium cantharidinate and vitamin B6 injection (Wang J. et al., 2018; He et al., 2021; Huang et al., 2023). However, CTD has strong toxic side effects, long-term clinical application will also lead to adverse reactions, and even death in severe cases (Zhang et al., 2020a; Jin et al., 2023), which greatly limit its further clinical application (Wang G. et al., 2018; Moya et al., 2014).

Numerous studies have shown that CTD can cause toxic damage to multiple organs, including the liver, kidneys, heart, lungs, gastrointestinal tract, bladder, and testes, with liver damage being the most severe (Wang J. et al., 2018; Naz et al., 2020; Chang et al., 2008; Huang et al., 2021; Xiao et al., 2023). CTD can cause punctate necrosis, vacuole, and edema of rat liver cells, mainly by interfering with lipid metabolism and the steroid hormone biosynthesis pathway (Zhang et al., 2020b). CTD may inhibit the hepatotoxicity induced by drug metabolism by inhibiting the expression of metabolic enzymes in the rat liver (Zhou et al., 2015). Zhu et al. (2019) found that 14 days after intragastric administration of CTD, 54 metabolites and 14 metabolic pathways were disordered, mainly by affecting amino acid and energy metabolism pathways, leading to hepatocyte apoptosis and necrosis. CTD has also been shown to induce cell damage in L-O2 cells with a “dose-time-toxicity” relationship. Dilation of the endoplasmic reticulum, autophagosomes, and apoptotic bodies has been observed under transmission electron microscopy, which further induced apoptosis by regulating oxidative stress, endoplasmic reticulum stress, and autophagy (Liu et al., 2020a). CTD can also induce hepatotoxicity by inhibiting the metabolism of cysteine, methionine, glutathione, glycine, serine, and threonine in L-O2 cells (Liu et al., 2020b). Additionally, we have previously shown that CTD can cause pathological damage and oxidative stress in the liver of mice, mainly by regulating glycerol phospholipid metabolism, the ABC transporter pathway, and choline metabolism (Huang et al., 2021). However, the detailed molecular mechanism of CTD-induced hepatotoxicity remains unclear.

DNA damage-induced transcription factor 4 (DDIT4), also known as DNA damage response-1 (REDD1) or dexamethasone-induced gene-2 (Dig2), is a recognized cellular stress protein that is highly expressed under conditions of hypoxia, DNA damage, and oxidative stress and can negatively regulate the activity of mammalian target of rapamycin (mTOR) protein (Ellisen, 2005; Pan et al., 2023). Many studies have shown that overexpression of DDIT4 can induce autophagy and apoptosis of nerve cells and

cardiomyocytes (Li et al., 2017; Huang et al., 2019; Li et al., 2023). DDIT4 plays an important role in autophagy and apoptosis in methamphetamine-induced cardiomyocyte injury and cerebral ischemia-reperfusion injury by regulating the mTOR pathway (Chen et al., 2016; Xu et al., 2021). However, its role in CTD-induced hepatotoxicity has not been reported.

In this study, we established CTD-induced liver injury models in mice and CTD exposure models in normal human L-O2 liver cells through *in vivo* and *in vitro* experiments, respectively, to investigate CTD-induced autophagy and apoptosis in liver cells. Exploring the important role of the DDIT4/mTOR pathway in CTD-induced liver injury and L-O2 cell injury in mice at the pathological, biochemical, molecular, and cellular levels will provide important theoretical references for further elucidating the mechanism of CTD-induced liver toxicity.

2 Materials and methods

2.1 Chemicals, animals, and treatment

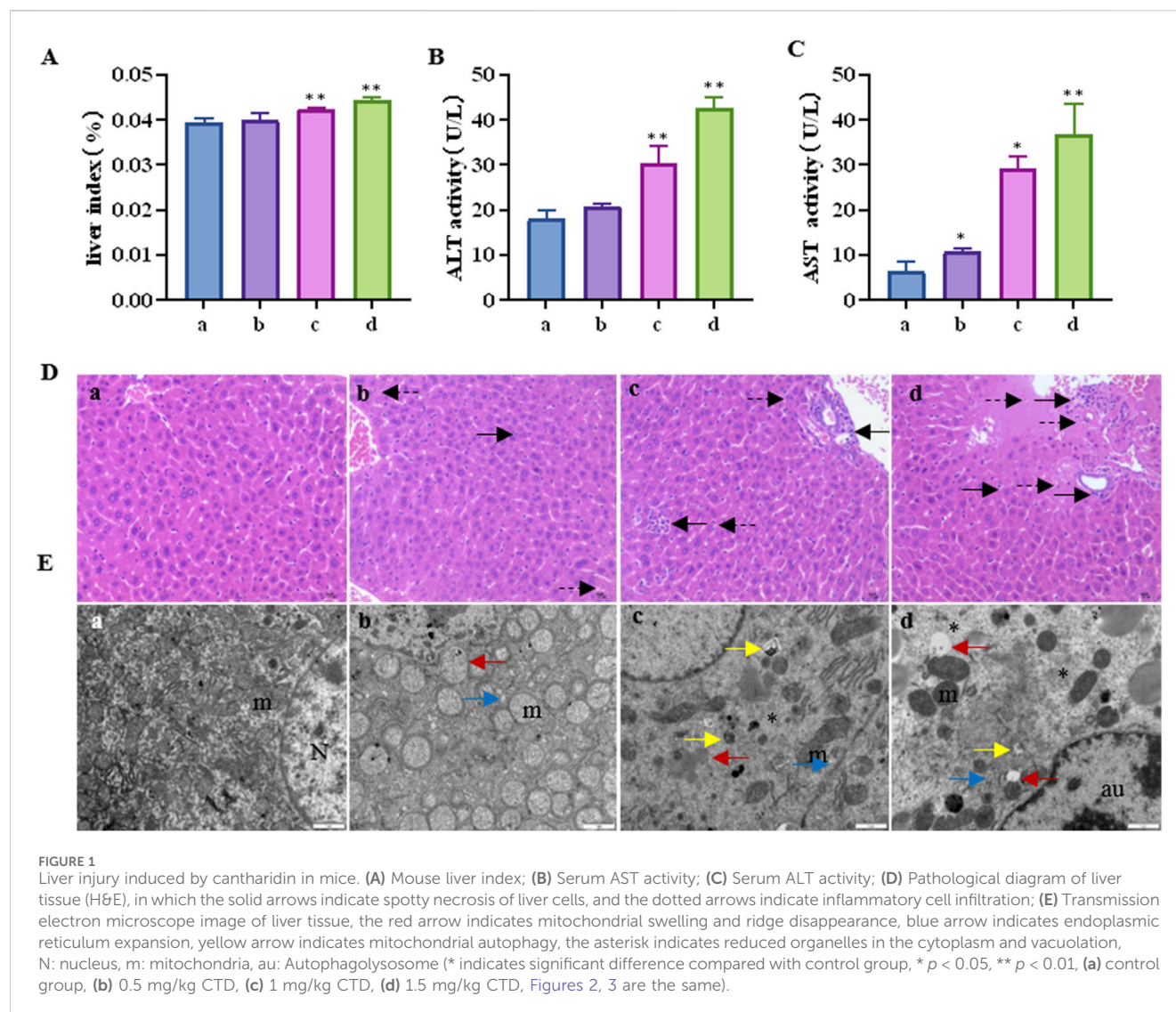
Cantharidin (purity $\geq 98\%$) was purchased from Sigma Company (USA). According to our previous method (Liu et al., 2023), a CTD suspension (4 mg/mL) was prepared with 0.5% sodium carboxymethyl cellulose solution and stored at 4°C for later use.

Forty SPF grade 6-week-old male Kunming mice, body weight 20 ± 2 g, were purchased from Changsha Tianqin Biotechnology Co., Ltd. (Hunan, China) (qualification certificate No: SCXK (Xiang) 2022-0011). The mice were kept in an aerated cage with an ambient temperature of $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$, relative humidity of $(55 \pm 5)\%$, and alternating white light/darkness for 12 h/12 h, and fed a standard diet and water. The animal experiment was approved by the Animal Experiment Ethics Committee of Guizhou University of Chinese Medicine (Approval No. 20230039).

After 1 week of adaptive feeding, the mice were randomly divided into the following four groups: the blank control group (CG), and low, medium, and high-dose CTD groups ($n = 10$ mice per group). CTD model mice were given 0.5, 1.0, and 1.5 mg/kg CTD solution, and the control group was given equal volume CMC-Na solution to establish the CTD-induced liver injury model. After 14 days of intragastric administration (once a day), the mice in each group were weighed and anesthetized with 0.5% sodium solution of pentobarbital, before collecting their orbital blood. The blood was left at room temperature for 30 min, before centrifuging at 3,000 g at 4°C for 10 min, and collecting the serum for later use. The animals were sacrificed by cervical dislocation and dissected under an ice bath, and the liver tissues were quickly separated, weighed, and recorded. Part of the liver tissues were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde solutions, and the remaining samples were first placed in liquid nitrogen and then transferred to -80°C for later use.

2.2 Liver index

The mouse liver index was calculated as = wet weight of liver (g)/last body weight (g) $\times 100\%$.



2.3 Detection of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels

The activity of ALT and AST enzymes in mouse serum was detected using kits purchased from Nanjing Jiancheng Biotechnology Research Institute (Jiangsu, China), according to the manufacturer's instructions.

2.4 Pathological observation and immunohistochemical staining of the liver tissue

The liver tissues fixed in paraformaldehyde for 24 h were dehydrated by gradient ethanol, transparent by xylene, embedded in paraffin, and then cut into 4- μ m thick sections, before staining with hematoxylin–eosin (HE), fixing with neutral gum tablets, and storing at room temperature. Histopathological observations were made under an optical microscope, followed by photographing.

Immunohistochemical staining was performed using the kit (Sangon Biotech, Shanghai, China), and the images were observed under an optical microscope. ImageJ (Java 1.8.0 by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) was used to quantify the positive expression rate of the DDIT4 protein (three slices were randomly selected for each animal, with 18 slides per group).

2.5 Cell culture

Cantharidin, 3-methyladenine (MCE, USA), and rapamycin (MCE, USA) were prepared into 100, 50, and 20 mM solutions with DMSO, respectively, and stored at -20°C for future use. Upon use, the corresponding culture medium was diluted to the required concentration. Human normal liver cells (L-O2) were purchased from Saibaikang (Shanghai) Biotechnology Co., Ltd. and were identified by STR (iCell-h054). After rehydration and passage, L-O2 cells were expanded and cultured under 37°C and 5% CO_2 saturation humidity.

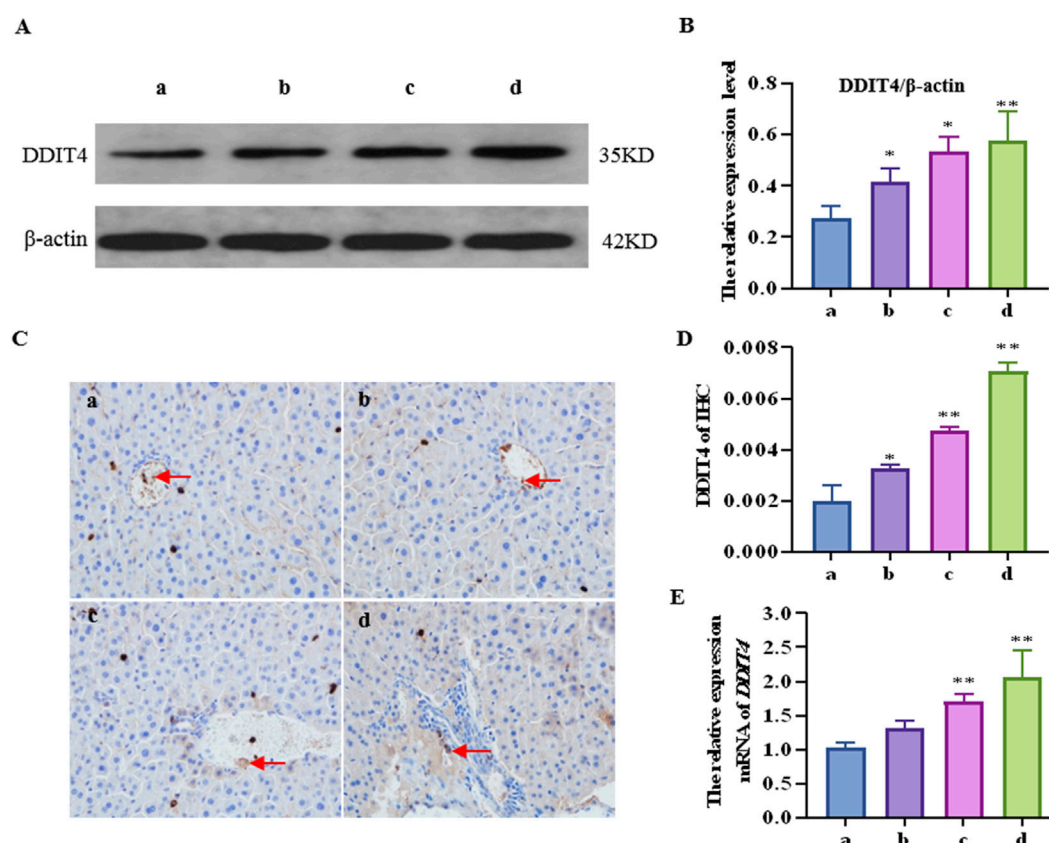


FIGURE 2
Effects of cantharidin on mRNA and protein expression of DDIT4 in mouse liver. (A) western blot gel electrophoresis of DDIT4, with β -actin as the internal reference; (B) Immunohistochemical map of DDIT4 protein, the brown area indicated by the arrow represents the positive rate; (C) Quantitative protein map by western blot; (D) Immunohistochemical quantitative map; (E) qRT-PCR diagram of DDIT4 gene, with β -actin as the internal reference gene.

2.6 Cell viability assay

L-O2 cells in the logarithmic growth stage and a good growth state were used for experiments. Briefly, 5×10^3 cells/well were inserted into a 96-well plate, and a blank group was set up and cultured overnight at 37°C. According to the different groups and cell treatment settings, the cells were treated separately and cultured in an incubator at 37°C and 5% CO₂. The groups were as follows: 1) normal cells; 2) 0.9375 μ mol/L CTD group; 3) 1.875 μ mol/L CTD group; 4) 3.75 μ mol/L CTD group; 5) 7.5 μ mol/L CTD group; 6) 15 μ mol/L CTD group; 7) 30 μ mol/L CTD group; and 8) 60 μ mol/L CTD group. L-O2 cells were treated with different concentrations of CTD for 6, 12, 24, and 36 h. After the specified time had elapsed, 10 μ L CCK-8 reagent was added to each well (Elabscience, China) and cultured at 37°C for 4 h. The OD₄₅₀ in each well was determined by enzyme-labeled assay.

2.7 qRT-PCR

After extracting total RNA from liver tissues (0.5, 1.0, and 1.5 mg/kg CTD exposure groups) or L-O2 cells (0, 7.5, 15, and 30 μ mol/L CTD treatment for 24 h) using TRIzol (Ambion, USA), HiScript[®] II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme,

China) was reverse transcribed into cDNA. According to the instructions of the SYBR Green Master Mix (Vazyme, China) assay kit, GAPDH was used as the internal reference gene to detect the relative DDIT4 mRNA expression level. Primer Premier 5.0 (Canada) was used to design the primer sequences, as shown in [Supplementary Table S1](#). The primers were synthesized by Beijing Qingke Biotechnology Co., Ltd. (China). The final data were quantitatively analyzed using the $2^{-\Delta\Delta C_t}$ method.

2.8 Cell transfection with siRNA

To knock down DDIT4, L-O2 cells were transfected with double-stranded siRNA (GenePharma, Shanghai, China). The sequence of siDDIT4 was as follows: sense, GUUUGUGUAUCU UACUGGUTT; antisense, ACCAGUAAGAUACACAAACTT. L-O2 cells were inoculated in 6-well plates, cultured in normal medium for 24 h, and then replaced with serum-free RPMI 1640 2 h before transfection. Transfection was performed according to the following experimental groups: 1) normal group; 2) interference negative control (siNC) group; 3) siDDIT4 group; 4) siDDIT4+Rap group; 5) siDDIT4+3-MA group; 6) CTD + siNC group; 7) CTD + siDDIT4 group; 8) CTD + siDDIT4+Rap group; and 9) CTD + siDDIT4+3-MA group. Following the manufacturer's

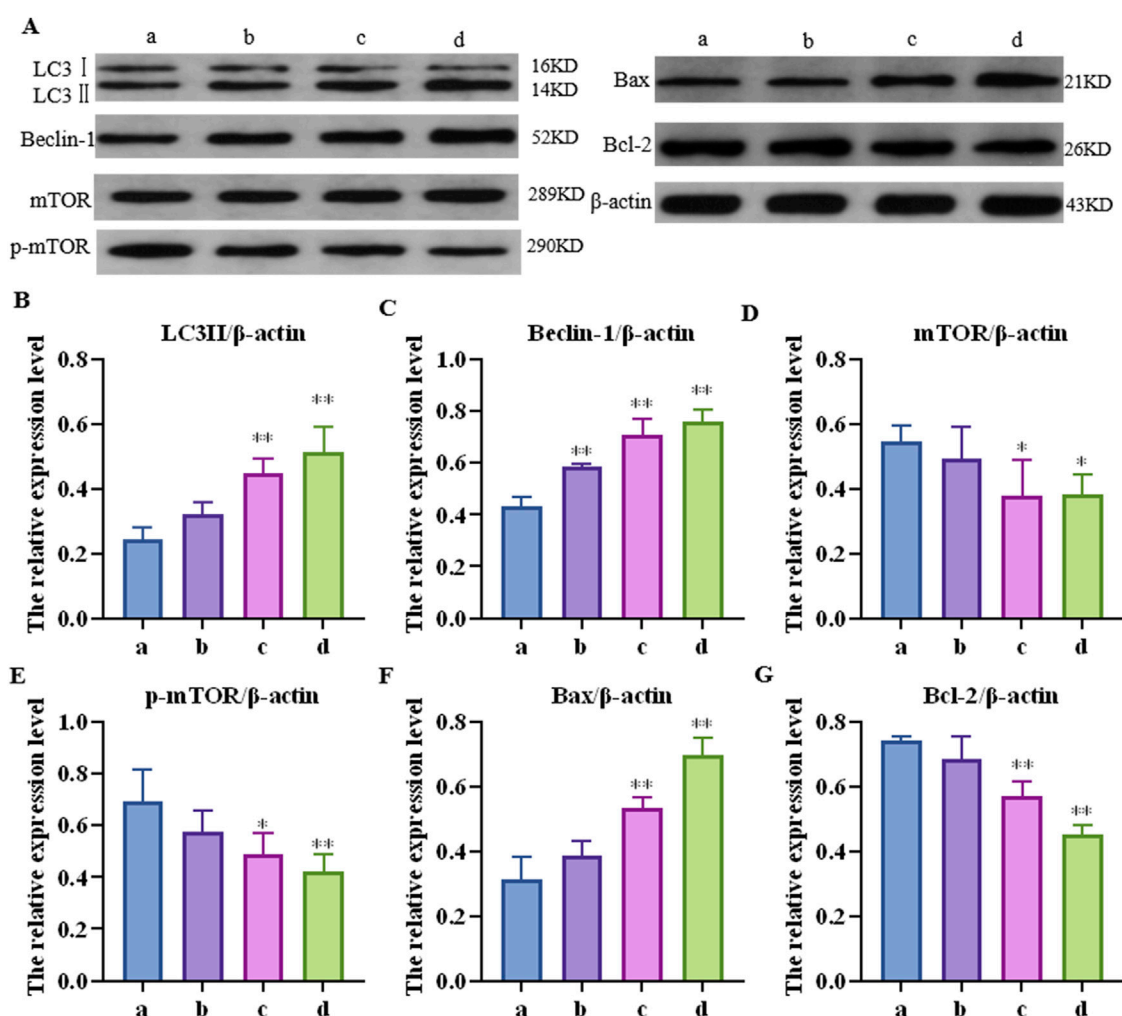


FIGURE 3 Effects of cantharidin on the expression of autophagy and apoptosis-related proteins in mouse liver. (A) western blot gel electrophoresis; (B–G): represents the relative expression of LC3II, Beclin-1, mTOR, p-mTOR, Bax and Bcl-2, respectively.

instructions, Lipofectamine 3,000 (Mei5 Biotechnology, Beijing, China) was used to transfect SiDDIT4 or siNC directly into target cells. According to the group, CTD was added for 12 h, and then Rap or 3-MA was added for 12 h.

2.9 Western blotting

RIPA protein lysis solution (Beyotime, China) was added to the processed mouse liver tissue or L-O2 cells, before placing them on ice for thorough grinding and lysis. Subsequently, the samples were centrifuged at 13,400 g at 4°C for 5 min, before collecting the supernatant and measuring the protein concentration using the BCA protein concentration assay kit (Beyotime, China). The tissue protein samples were subjected to SDS-PAGE gel electrophoresis and membrane transfer, before sealing with 5% skimmed milk powder for 2 h. The following antibodies were dripped: DDIT4 (1:1000, A14135, Abclonal, China), Beclin-1 (1:1000, A7353, Abclonal, China), LC3 (1:1000, AF5402, Affinity, USA), mTOR (1:1000, AF6308, Affinity, USA), p-mTOR (1:1000, AF3308,

Affinity, USA), Bax (1:1000, AF0120, Affinity, USA), Bcl-2 (1:1000, AF6139, Affinity, USA) β-Actin (1:1000, BM0627, Bosterbio, China), and GAPDH (1:1000, AB-P-R 001, Goodhere, China), where β-Actin and GAPDH were used as internal references. The antibodies were incubated overnight at 4°C, and then hybridized with HRP-labeled goat anti-rabbit IgG secondary antibody (1:10,000, BA1054, Bosterbio, China). Excess antibody was washed off with TBST, and ECL chemiluminescence was used for development exposure. The film was dried and scanned, and the grayscale value of the film was analyzed and photographed using Image Pro Plus.

2.10 Flow cytometry

L-O2 cells in each group were inoculated into 6-well plates and incubated at 37°C overnight, before discarding the medium and washing the adherent cells twice with phosphate buffered saline (PBS). To obtain the suspension, the cells were digested with 0.25% trypsin without EDTA, transferred to a new EP tube, centrifuged at

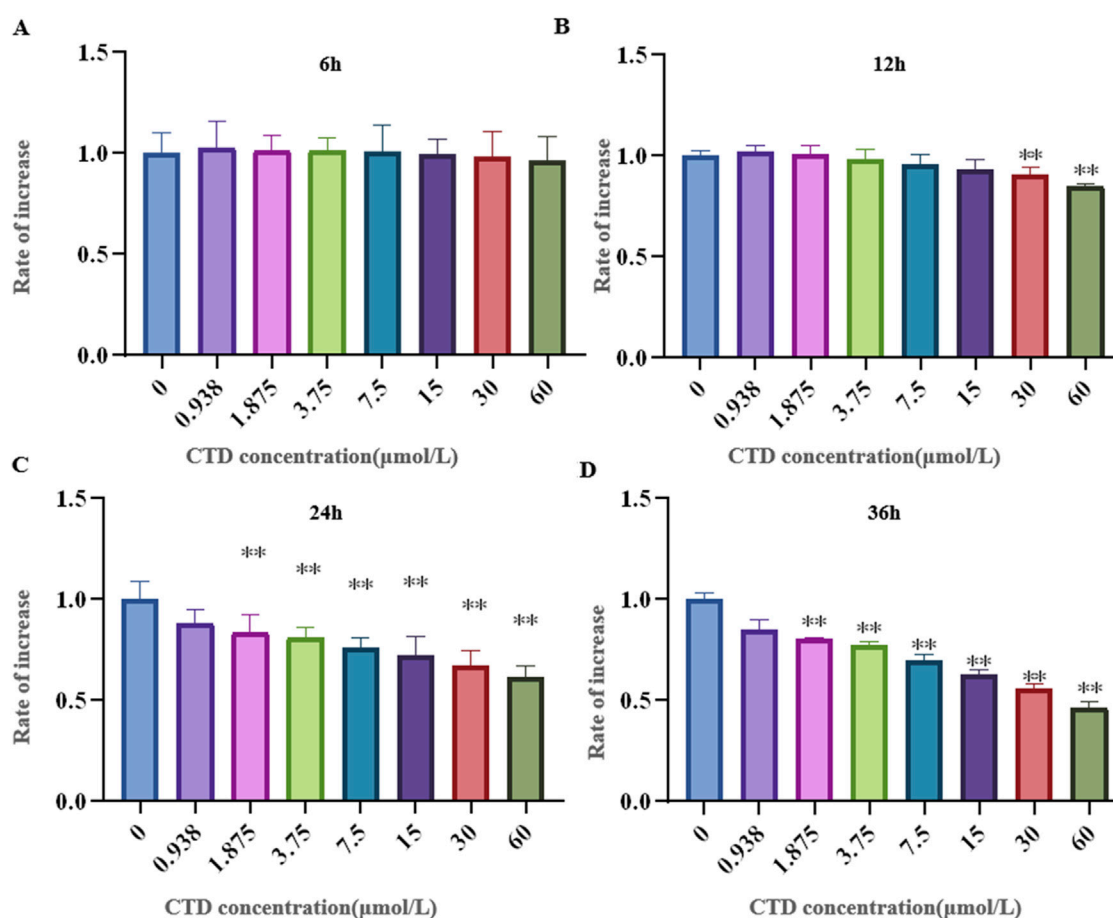


FIGURE 4
Effects of CTD on L-O2 cytotoxicity. (A–D): CCK-8 detected the activity of L-O2 cells after CTD exposure at different times, and a–h represented 0, 0.9375, 1.875, 3.75, 7.5, 15, 30 and 60 μmol/L CTD treatment groups, respectively. (* indicated a significant difference compared with the control group, * $p < 0.05$, ** $p < 0.01$, same in Figures 5, 6)

1000 g at 4°C for 5 min, the supernatant was discarded, and the cells were rinsed twice with PBS at 1000 g for 5 min. Subsequently, apoptosis was detected using the AnnexinV-APC/7-AAD apoptosis detection kit (Keygen Biotech, KGA1026, China), and on-machine analysis was performed by flow cytometry (Beckman Coulter, cytoFLEX, USA).

2.11 Transmission electron microscopy

Small pieces of liver tissue or L-O2 cells were fixed with 2.5% glutaraldehyde. The steps of cleaning, fixation, dehydration, polymerization, continuous ultra-thin sectioning, and staining followed the method described in our previous publication (Tang et al., 2018). Transmission electron microscopy (Hitachi, ht7800/ht7700, Japan) was used to obtain images at 100 kV.

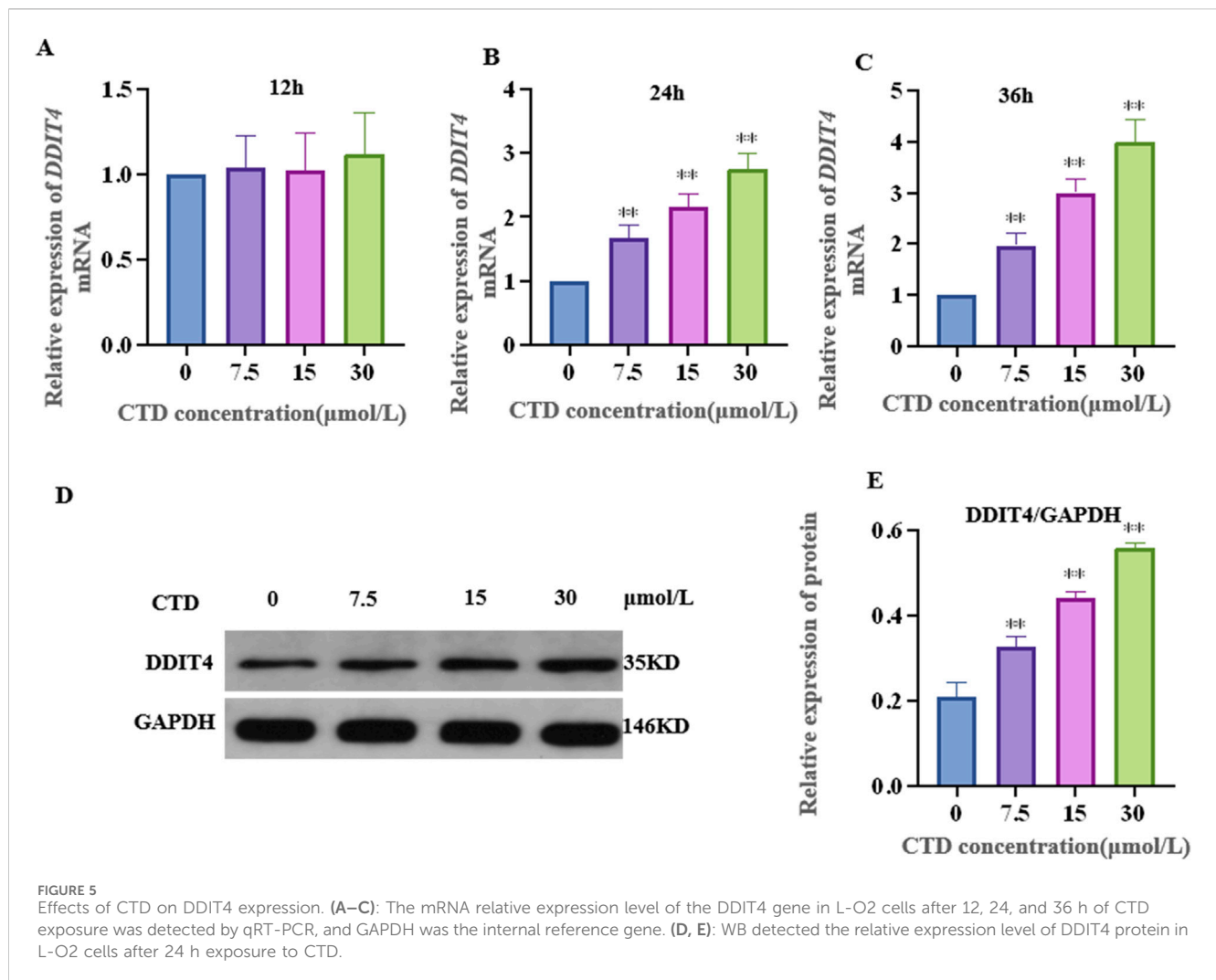
2.12 Hoechst 33,342 staining

siNC- or SIDDIT4-transfected L-O2 cells were transfected with the GFP-LC3 plasmid (provided by Hanshenti Technology, Inc.) using

Lipofectamine 2000 reagent (Invitrogen, USA). L-O2 cells were inoculated on a 24-well plate and allowed to attach. Subsequently, the cells were soaked with PBS three times, for 3 min each time, before fixing with 4% paraformaldehyde drops for 15 min. The slides were soaked with PBS three times, for 3 min each time, before using absorbent paper to blot the PBS, and adding Hoechst 33,342 dye solution to the slides (Beyotime, China). After incubation at room temperature for 5 min, the slides were soaked with PBS three times, for 3 min each time, the liquid on the slides was dried with absorbent paper, and the slides were sealed with a sealing solution containing the anti-fluorescence quench agent Southernbiotech (USA). The images were observed and collected under a confocal laser microscope (Nikon C2, Japan).

2.13 Statistical analysis

All data were statistically analyzed using SPSS 28.0 (SPSS Inc., Chicago, IL). Dunnett's t -test was used to compare the multiple groups, and one-way analysis of variance (ANOVA) was used to analyze the statistical differences among the multiple groups. The values are expressed as the mean $\pm$ standard deviation, and p -values < 0.05 were considered to indicate statistical significance.



3 Results

3.1 CTD-induced liver injury in mice

The liver index of the mice is shown in Figure 1A. Compared to the control group, the liver index showed a tendency to increase with increasing CTD exposure concentration, especially in the 1 mg/kg and 1.5 mg/kg CTD exposure groups ($p < 0.05$). The serum liver function indices (AST and ALT activities) are shown in Figures 1B, C. Compared to the control group, AST activity was significantly increased. In the low, medium, and high concentrations, CTD groups were increased by 1.67 ($p > 0.05$), 4.55 ($p < 0.01$), and 5.74 ($p < 0.01$) times, respectively. ALT activity was increased by 1.16 ($p > 0.05$), 1.69 ($p < 0.01$), and 2.38 ($p < 0.01$) times. Histopathological examination of the livers of mice showed spotty necrosis and inflammatory cell infiltration (Figure 1D). Ultrafine structure observation showed that after CTD exposure, mitochondrial swelling and ridge disappearance, endoplasmic reticulum expansion, mitochondrial autophagosomes, and lysosomes appeared in the cytoplasm, and even reduced organelles and cellular vacuolation in the cytoplasm at high concentrations (Figure 1E). The pathological damage became more severe with the increase in CTD exposure concentration.

3.2 CTD leads to increased DDIT4 mRNA and protein expression levels in mouse liver tissue

The results of western blotting and immunohistochemistry (Figures 2A–D) showed that the DDIT4 protein expression levels in all concentration groups were significantly increased after CTD exposure ($p < 0.05$), particularly at high concentrations ($p < 0.01$). Moreover, the qRT-PCR results showed that compared to the control group, the mRNA relative expression level of the DDIT4 gene in the medium- and high-concentration CTD-exposed groups was significantly increased ($p < 0.01$) (Figure 2E).

3.3 CTD causes increased autophagy and apoptosis levels in the mouse liver

Further detection results of the mTOR pathway, autophagy-related proteins (LC3II and Beclin-1), and apoptosis key proteins (Bax and Bcl-2) in the mouse liver are shown in Figure 3. Compared to the control group, the relative protein expression of LC3-II in each concentration group was upregulated by 1.33 ($p > 0.05$), 1.84

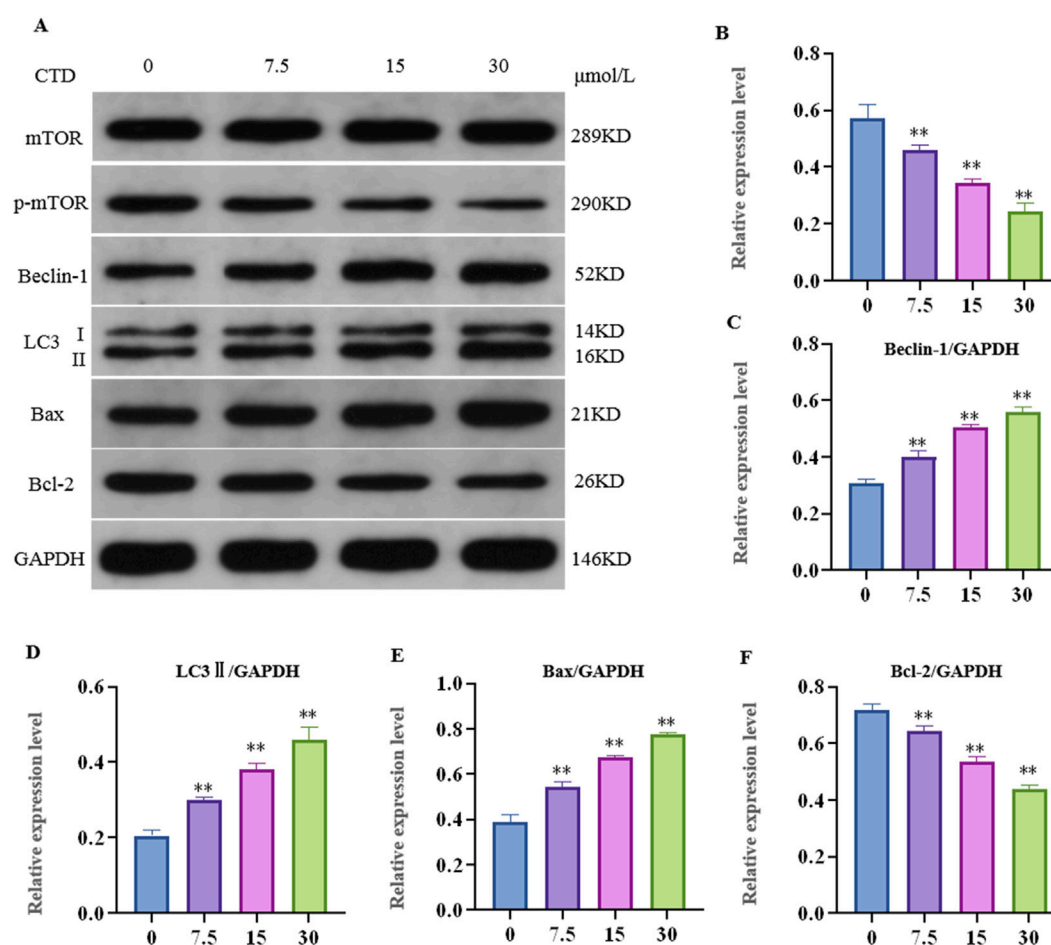


FIGURE 6
Effects of cantharidin on autophagy and apoptosis-related protein expression in L-O2 cells. (A) western blot gel electrophoresis; (B–F): indicates the relative protein expression of p-mTOR, LC3II, Beclin-1, Bax, and Bcl-2, respectively.

($p < 0.05$), and 2.10 ($p < 0.01$) times, respectively; Beclin-1 was upregulated 1.35 ($p < 0.01$), 1.84 ($p < 0.01$), and 2.10 ($p < 0.01$) times, respectively; mTOR was downregulated 1.31 ($p > 0.05$), 1.44 ($p < 0.05$), and 1.44 ($p < 0.01$) times, respectively; p-mTOR was downregulated 1.13 ($p > 0.05$), 1.35 ($p < 0.05$), and 1.60 ($p < 0.01$) times, respectively; Bax was increased by 1.23 ($p > 0.05$), 1.71 ($p < 0.01$), and 2.23 ($p < 0.01$) times, respectively; and Bcl-2 was downregulated by 1.08 ($p > 0.05$), 1.30 ($p < 0.01$), and 1.65 ($p < 0.01$) times, respectively.

3.4 CTD exhibited toxicity to L-O2 cells and increased DDIT4 expression

The results of the CCK-8 assay and L-O2 cell viability experiments are shown in Figure 4. Compared to the control group, after 6 h exposure to CTD, the cell viability of each concentration group showed no significant change ($p > 0.05$). After 12 h, with the exception of the 30 and 60 $\mu\text{mol/L}$ CTD exposure groups, the cell viability decreased significantly ($p < 0.01$), while no significant changes were observed in the other groups ($p > 0.05$). After 24 and 36 h, with the exception of the

0.9375 $\mu\text{mol/L}$ CTD exposure group, the cell viability was significantly decreased in all other groups ($p < 0.01$). According to the results of CCK-8, we detected the relative DDIT4 gene mRNA expression in L-O2 cells after exposure to CTD (15, 30, and 60 $\mu\text{mol/L}$) (12, 24 and 36 h) by qRT-PCR. The results showed that, compared to the control group, after exposure to CTD for 12 h, there was no significant change in the DDIT4 gene mRNA expression level in all concentration groups ($p > 0.05$) (Figure 5A). Moreover, after 24 and 36 h, the mRNA expression level of the DDIT4 gene in all concentration groups was significantly increased ($p < 0.01$) (Figures 5B, C). We further determined the relative expression level of DDIT4 protein after 24 h exposure to CTD, and the results showed that the DDIT4 protein expression in each concentration group was significantly increased compared to that in the control group ($p < 0.01$) (Figures 5D, E).

3.5 CTD increased autophagy and apoptosis of L-O2 cells

After 24 h of CTD exposure, the results of the experiments pertaining to the mTOR pathway, autophagy, and apoptosis-related

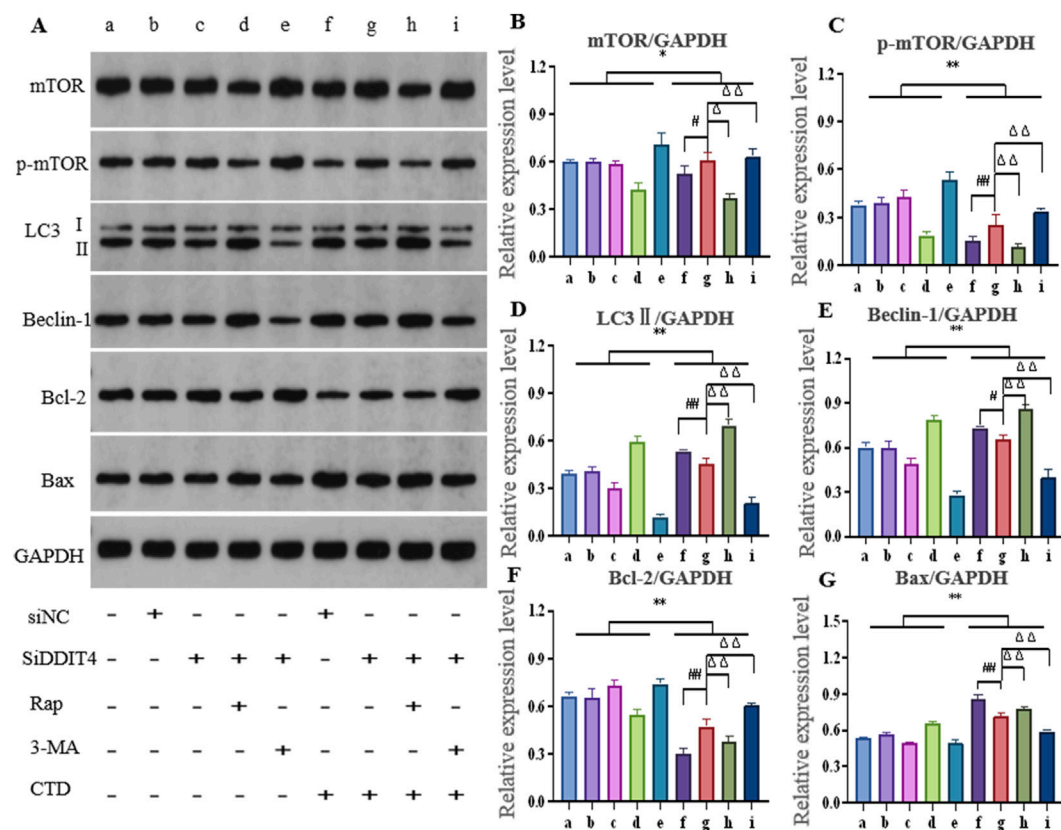


FIGURE 7

Effects of cantharidin on autophagy and apoptosis-related protein expression in L-O2 cells. (A) western blot gel electrophoresis; (B–G): Histogram of protein relative expression of mTOR, p-mTOR, LC3II, Beclin-1, Bcl-2 and Bax, respectively (Control: b, c, d, e; CTD: f, g, h, i; * for vs. Control, * $p < 0.05$, ** $p < 0.01$; # indicates vs. CTD + SiNC, ## $p < 0.01$; Δ represents vs. CTD + SiDDIT4, ΔΔ $p < 0.01$, same as Figure 8).

proteins in L-O2 cells are shown in Figure 6. Compared to the control group, the relative expression levels of p-mTOR and Bcl-2 proteins in the 7.5, 15 and 30 $\mu\text{mol/L}$ CTD exposure groups were significantly decreased ($p < 0.01$), while those of LC3II, Beclin-1, and Bax were significantly increased ($p < 0.01$).

3.6 Effects of DDIT4 knockdown and autophagy activation/inhibition on autophagy and L-O2 cell apoptosis

RNAi was used to knock down the transcription and translation of the DDIT4 gene in cells, and western blotting was used to detect the expression of the DDIT4 protein. The results showed that the interference sequence targeting L-O2 cells could effectively knock down the expression of DDIT4 ($p < 0.01$). We selected the sequence with the highest knockdown for subsequent experiments (Supplementary Figure S1).

3.6.1 Western blot to detect autophagy and apoptosis of L-O2 cells

After knocking down DDIT4 and using the autophagy activator Rap or inhibitor 3-MA, the expression of autophagy- and apoptosis-related proteins in L-O2 cells is shown in Figure 7. The relative expression levels of mTOR ($p < 0.05$), p-mTOR ($p < 0.01$), and Bcl-2 ($p < 0.01$) in the

CTD-exposed groups were significantly decreased, whereas those of LC3-II, Beclin-1, and Bax were significantly increased ($p < 0.01$). After DDIT4 deletion, compared to the CTD + SiNC group, the relative expression levels of mTOR ($p < 0.05$), p-mTOR ($p < 0.01$), and Bcl-2 ($p < 0.01$) in the CTD + SiDDIT4 group were significantly increased, while those of LC3-II ($p < 0.01$), Beclin-1 ($p < 0.05$), and Bax ($p < 0.01$) were significantly decreased. After treatment with Rap, compared to the CTD + SiDDIT4 group, the relative expressions of mTOR, p-mTOR, and Bcl-2 proteins in the CTD + SiDDIT4+Rap group were significantly decreased ($p < 0.01$), whereas those of LC3-II, Beclin-1, and Bax were significantly increased ($p < 0.01$). After 3-MA treatment, compared to the CTD + SiDDIT4 group, the relative expressions of p-mTOR and Bcl-2 proteins in the CTD + SiDDIT4+ 3-MA group were significantly increased ($p < 0.01$), whereas LC3-II, Beclin-1, and Bax were significantly decreased ($p < 0.01$).

3.6.2 Autophagy of L-O2 cells detected by confocal microscopy

The expression level of LC3-II in L-O2 cells was further detected by Hoechst 33,342 staining, with the results shown in Figures 8A, B. Compared to the control groups, LC3-II expression in the CTD-exposed groups was significantly increased ($p < 0.01$). After DDIT4 deletion, LC3II expression in the CTD + SiDDIT4 group was significantly decreased compared to that in the CTD + SiNC group ($p < 0.01$). After treatment with Rap, LC3-II expression in the CTD +

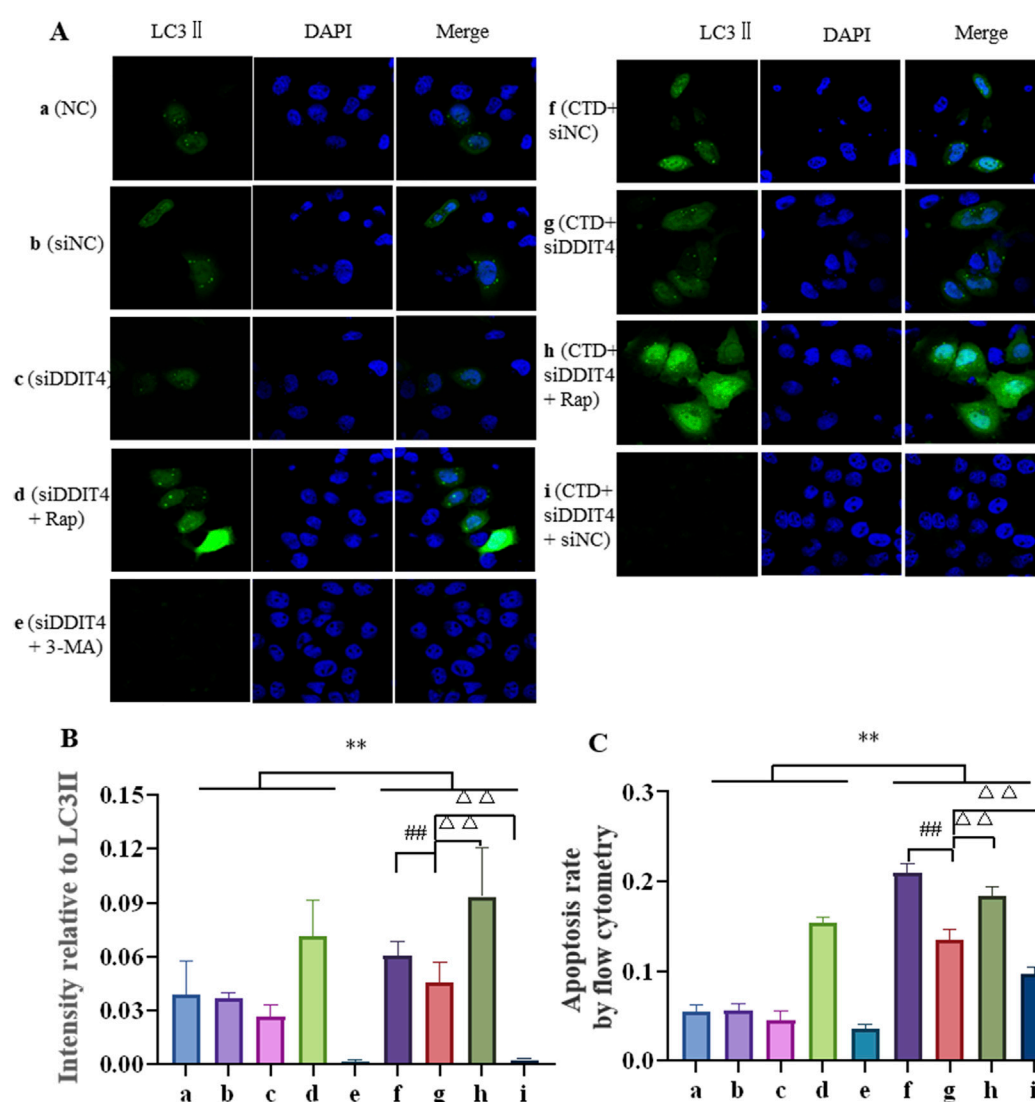


FIGURE 8
Hoechst33342 staining of LC3II (A, B), and the apoptosis level of L-O2 cells was detected by flow cytometry (C).

SiDDIT4+Rap group was significantly higher than that in the CTD + SiDDIT4+Rap group ($p < 0.01$). After 3-MA treatment, LC3-II expression in the CTD + SiDDIT4+3-MA group, LC3II expression in the CTD + SiDDIT4+3-MA group was significantly decreased ($p < 0.01$).

3.6.3 Flow cytometric detection of L-O2 cell apoptosis

Flow cytometry was further used to detect L-O2 cell apoptosis, with the results shown in Figure 8C and Supplementary Figure S2. Compared to the control groups, the apoptosis rate of the CTD-exposed groups was significantly increased ($p < 0.01$). After DDIT4 knockdown, compared to the CTD + SiNC group, the apoptosis rate of the CTD + SiDDIT4 group was significantly decreased ($p < 0.01$). Compared to the CTD + SiDDIT4+Rap group, the apoptosis rate of the CTD + SiDDIT4+Rap group was significantly increased ($p < 0.01$). After 3-MA treatment, compared to the CTD + SiDDIT4 group, the apoptosis rate of the CTD + SiDDIT4+3-MA group was significantly decreased ($p < 0.01$).

3.6.4 Transmission electron microscopy observation of L-O2 cell autophagy

In each group, autophagy was observed by transmission electron microscopy, with the results shown in Figure 9. Mitochondrial autophagy was observed in the NC group and all control groups. After CTD exposure, the mitochondrial autophagy levels increased to a certain extent, whereas Rap treatment significantly increased mitochondrial autophagy. In contrast, mitochondrial autophagy was significantly inhibited after 3-MA treatment. The observed mitochondrial autophagy results are consistent with the western blotting and immunofluorescence results.

4 Discussion

Due to the strong killing effect of its main active ingredient CTD on various stubborn tumors, the traditional Chinese medicine Mylabris has been developed into numerous anti-tumor

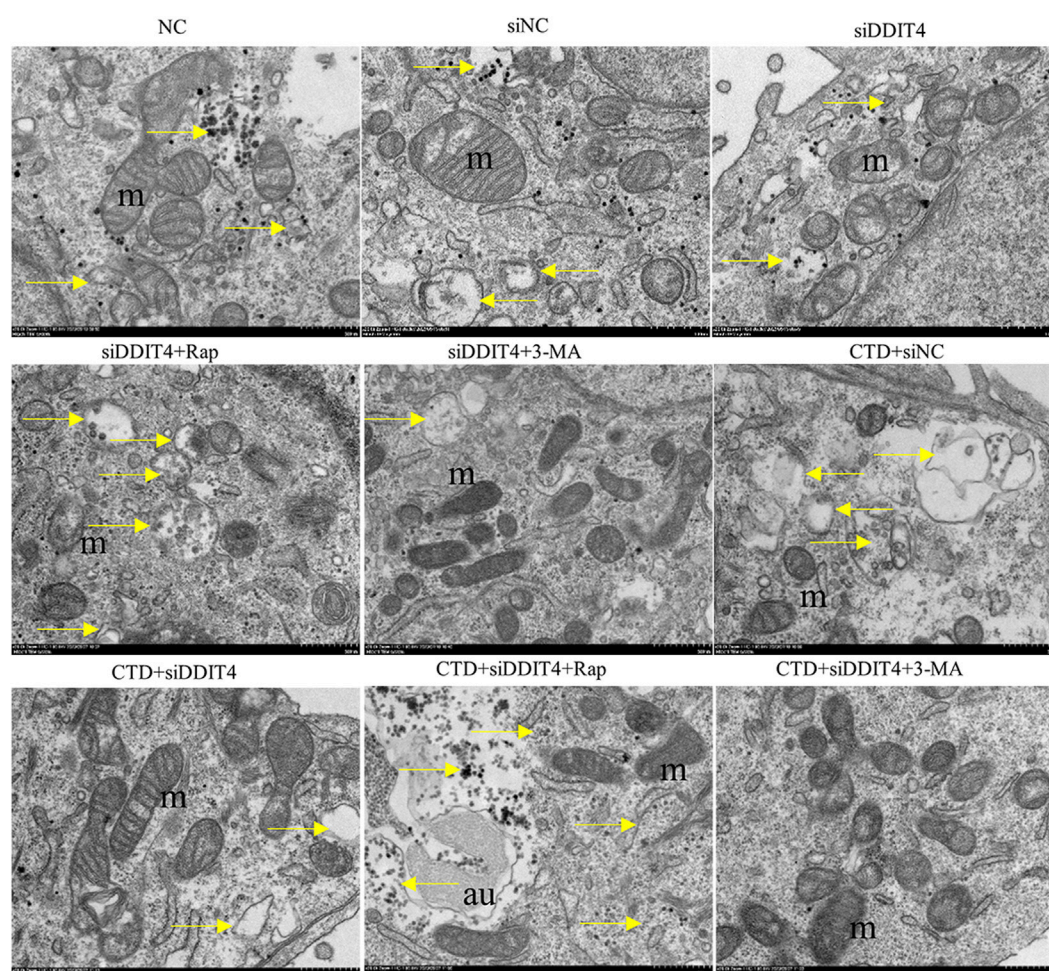
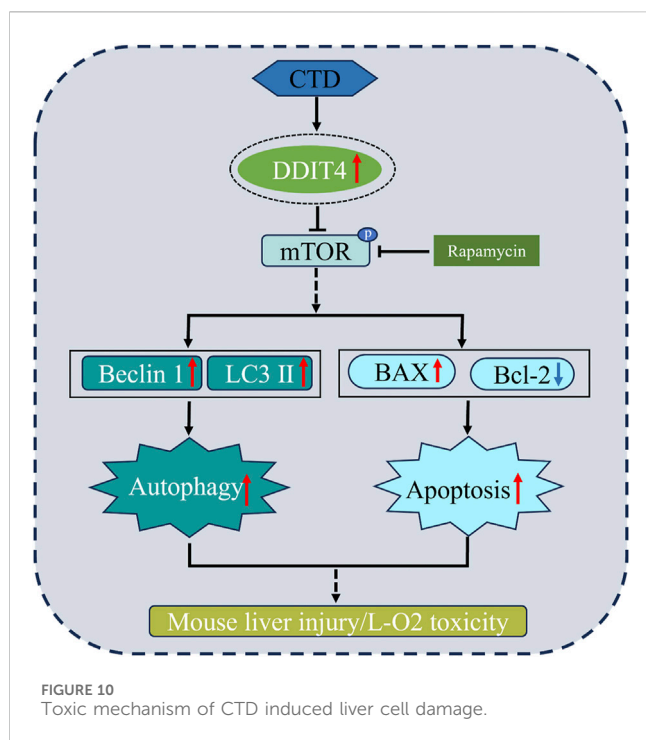


FIGURE 9
Transmission electron microscopy of L-O2 cells. The yellow arrows in the figure indicate mitochondrial autophagy, m: mitochondria, au: autophagy lysosomes.

traditional Chinese medicines for the market, including Aidi Injection, Sodium Cantharidinate Vitamin B6 Injection, and Compound Cantharidin Capsules (Liu et al., 2020b; He et al., 2021). However, the therapeutic dose of CTD is very close to its toxic dose, and long-term use can lead to serious liver and kidney toxicity (Jin et al., 2023). A recent review summarized the current mechanisms of CTD-induced hepatotoxicity, indicating that CTD leads to varying degrees of liver damage by activating endogenous and exogenous pathways, resulting in hepatocyte apoptosis and autophagy (Jin et al., 2023). However, the mechanism of CTD-induced hepatotoxicity has not been fully elucidated. In this study, we investigated the important role of the DDIT4/mTOR signaling pathway in CTD-induced autophagy and apoptosis of liver cells, providing an important theoretical basis for a better understanding of the mechanism of CTD-induced hepatotoxicity.

It is well known that an increase in liver index and serum AST and ALT levels are important indicators for the evaluation of liver injury. In our study, the liver index and serum ALT and AST levels of mice increased significantly after exposure to different concentrations of CTD and showed dose dependence. Moreover, obvious pathological damage of mouse liver tissue was observed by

HE staining and transmission electron microscopy, suggesting that CTD can cause liver injury in mice, confirming successful generation of the animal model. Through transmission electron microscopy, we also observed that liver cell damage is closely related to mitochondrial autophagy. Previous studies have shown that CTD can induce autophagy and apoptosis in L-O2 cells (Liu et al., 2020a). We further detected autophagy-related proteins (Beclin-1 and LC3-II) and apoptosis-related proteins (Bax and Bcl-2) in the mouse liver. Beclin-1, also known as the autophagy-related gene (Atg) 6, is a recognized autophagy regulator and a key component in inducing autophagosome formation and lysosomal biogenesis (Fekadu and Rami, 2016). LC3-II is an important marker molecule of autophagosomes. During autophagy, LC3-I is modified and processed by ubiquitin-like systems, including Atg7 and Atg3, producing LC3-II, with a molecular weight of 14 kDa. The LC3-II content is directly proportional to the degree of autophagy (Galluzzi and Green, 2019), and enhanced expression of Beclin-1 and LC3-II is necessary for autophagy (Wang, 2008). In our study, with an increase in the CTD exposure concentration, both Beclin-1 and LC3-II showed significant increases, indicating that CTD can induce autophagy in the mouse liver. Related studies have shown



that the activation of Bax is a key event in the occurrence of apoptosis (Liu et al., 2011), whereas BCL2 can exert anti-apoptotic effects by inhibiting the release of cytochrome C and apoptosis initiation factors (Schultz and Harrington, 2003). Our results revealed that the expression of Bax protein was increased and that the expression of Bcl-2 was inhibited after CTD exposure, indicating that CTD caused apoptosis in the mouse liver. Our results are consistent with those of Liu et al. (2020a), who reported that CTD can induce autophagy and apoptosis of L-O2 cells *in vitro*.

mTOR is the main regulator of cell growth, proliferation, and metabolism, as well as being an inhibitor of autophagy induction (Li et al., 2012). Research has shown that mTOR signaling is significantly inhibited in liver injury induced by ischemia-reperfusion, carbon tetrachloride (CCl₄), and acetaminophen (Rao et al., 2017; Gao et al., 2020; Fang et al., 2023). In our study, both mTOR and p-mTOR were significantly downregulated in the mouse liver after CTD exposure, indicating that CTD could inhibit the mTOR pathway. It is well known that inhibition of the mTOR pathway promotes autophagy, while related studies have shown that inhibition of mTOR also promotes apoptosis (Wang et al., 2022; Xie et al., 2023; Kumari et al., 2023). In terms of how mTOR is inhibited in CTD-induced liver autophagy and apoptosis, previous studies have found that DDIT4, a negative regulatory protein of mTOR, increases autophagy and apoptosis by inhibiting mTOR phosphorylation (Chen et al., 2016; Xu et al., 2021). DDIT4, as a stress protein, exists at low levels in most cells, including liver cells, and its expression is closely related to various cellular injuries (Chen et al., 2023). In non-alcoholic fatty liver disease, liver fibrosis, methionine-choline-deficient diet induced steatotic liver injury, bile duct ligation surgery induced cholestatic liver injury, and in CCl₄ injection induced hepatotoxic injury, abnormal increase in DDIT4 expression (Dumas et al., 2020; Cho et al., 2021; Li et al., 2021). In our study, we also detected the expression levels of DDIT4 in the liver after CTD exposure. The results of qPCR,

immunohistochemistry, and western blot showed that CTD exposure led to an abnormal increase in DDIT4 mRNA and protein expression. These results suggest that the CTD-induced autophagy and apoptosis of hepatocytes may be related to the upregulation of DDIT4 and inhibition of the mTOR pathway. Therefore, we speculate that the DDIT4/mTOR pathway plays an important role in CTD-induced liver injury.

To further explore the important role of the DDIT4/mTOR pathway in CTD-induced hepatocyte autophagy and apoptosis, we established a toxic model of L-O2 cells induced by CTD *in vitro*. The results of the CCK-8 assay showed that CTD induced toxicity in L-O2 cells. According to the cell viability results after exposure to different concentrations of CTD for 6, 12, 24, and 36 h, and combined with the results of qPCR and western blotting for DDIT4, we selected CTD exposure concentrations of 7.5, 15, and 30 μmol/L and an exposure time of 24 h for follow-up experiments. Our results revealed that the DDIT4 protein expression in L-O2 cells also increased significantly after CTD exposure. The results of further detection of the mTOR pathway, autophagy, and apoptosis-related proteins in L-O2 cells were consistent with those in mice *in vivo*. Therefore, we speculate that the mechanism of CTD-induced autophagy and apoptosis in L-O2 cells may be related to the DDIT4/mTOR pathway.

To verify our hypothesis, we used RNAi to knock down the expression of DDIT4 and detected the expression of the mTOR pathway, autophagy, and apoptosis-related proteins. The results of western blot, flow cytometry, immunofluorescence, and transmission electron microscopy showed that knocking down DDIT4 expression significantly increased the expression of mTOR and p-mTOR and significantly inhibited CTD-induced autophagy and apoptosis of L-O2 cells. Furthermore, CTD-induced liver cell autophagy and apoptosis was increased after activating the mTOR pathway with the autophagy agonist Rap. The use of the autophagy inhibitor 3-MA to inhibit the mTOR pathway can alleviate CTD-induced liver cell autophagy and apoptosis. Taken together, these results suggest that CTD-induced autophagy and apoptosis in L-O2 cells are achieved through the DDIT4/mTOR pathway.

This study has some limitations that warrant discussion: 1) our *in vivo* experiments only positively prove that CTD-induced liver injury is related to autophagy and apoptosis of the DDIT4/mTOR pathway, while there remains a lack of negative evidence, such as verification at the gene knockout animal level; 2) there is insufficient evidence to prove that autophagy and apoptosis are the main mechanisms of CTD-induced hepatocyte injury; and 3) it remains unclear how CTD regulates the upregulation of DDIT4 expression and leads to autophagy and apoptosis of hepatocytes. We plan to conduct further research on these aspects in the future.

5 Conclusion

In summary, through *in vivo* and *in vitro* cell experiments, we proved that the molecular mechanism of liver injury and L-O2 cytotoxicity in mice induced by CTD may be mediated through increasing DDIT4 expression levels, thereby inhibiting mTOR phosphorylation and increasing autophagy and apoptosis. Autophagy and apoptosis of hepatocytes induced by CTD could

be alleviated to a certain extent by knocking down DDIT4 or inhibiting the mTOR pathway (Figure 10).

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repository and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The animal study was approved by the Animal Experiment Ethics Committee of Guizhou University of Chinese Medicine (Approval No. 20230039). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

WT: Writing–review and editing, Writing–original draft, Project administration, Funding acquisition, Data curation. YP: Writing–original draft, Software, Methodology, Formal Analysis. CZ: Writing–review and editing, Conceptualization, Validation. DL: Writing–review and editing, Resources, Investigation. FP: Writing–original draft, Resources, Methodology. QS: Writing–review and editing, Investigation. YX: Writing–review and editing, Methodology, Data curation.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work

was supported by the National Natural Science Foundation of China (Grant no. 82060753).

Acknowledgments

We thank International Science Editing for language editing. The authors would also like to thank the editors and reviewers for their valuable comments and suggestions.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2024.1480512/full#supplementary-material>

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RECEIVED 11 May 2024

ACCEPTED 06 December 2024

PUBLISHED 18 December 2024

CITATION

Long L, Jia Z and Liu T (2024) Association between urinary arsenic and hearing threshold shifts in adults in the United States, National Health and Nutrition Examination Survey, 2015–2016. *Front. Public Health* 12:1431122. doi: 10.3389/fpubh.2024.1431122

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Association between urinary arsenic and hearing threshold shifts in adults in the United States, National Health and Nutrition Examination Survey, 2015–2016

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Background: Hearing loss (HL) is a common sensory disorder in humans. Studies on the relationship between arsenic, which is a highly toxic and widely distributed heavy metal with a health risk to humans, and hearing status in humans are contradictory and mostly focused on people living in arsenic-contaminated areas. This study investigated the association between urinary arsenic levels and hearing threshold shifts in the general population in the United States.

Methods: Overall, 1,017 adults (aged 20–69 years) from the National Health and Nutrition Examination Survey (2015–2016) were included in this study. HL was defined as pure-tone average (PTA) ≥ 20 dB at frequencies 500, 1,000, 2,000, and 4,000 Hz in the better-hearing ear. Total urinary arsenic (uAs) and dimethylarsinic acid (uDMA) levels were analyzed. Multivariate linear regression analyses and smooth curve fitting were performed to evaluate the correlations between uDMA, uAs, and low-, speech-, and high-frequency hearing levels.

Results: The mean age of the participants was 42.13 ± 13.66 years, including weighted 48.67% men and 12.88% participants with sensorineural HL. After adjusting for potential confounders in the multivariate linear regression model, higher uDMA levels were significantly associated with poor low-, and speech-frequency PTAs, with no differences among participants by age or sex. Smooth curve fitting indicated a nonlinear relationship between uAs and high-frequency PTA hearing threshold shifts. The uAs levels were positively associated with high-frequency PTA until the turning point of 1.54 (adjusted β 4.53, 95% CI 1.16, 7.90; $p = 0.0085$), beyond which this association was not observed (adjusted β -0.43 , 95% CI -1.57 , 0.71; $p = 0.4600$).

Conclusion: We found positive associations between urinary arsenic metabolites uDMA, uAs levels and poor hearing threshold shifts in US adults. This study provides new evidence for the association between arsenic exposure and auditory function.

KEYWORDS

arsenic metabolites, hearing threshold shift, National Health and Nutrition Examination Survey, cross-sectional study, adults

1 Introduction

Hearing loss (HL) is one of the most common sensory disabilities in humans, affecting over 1.5 billion people globally. An estimated 430 million people have moderate or higher severity HL in the better-hearing ear. HL has lifelong ramifications, including adverse health and social and economic development, which can negatively affect the development of language and speech during childhood, education, communication, cognition, employment, and mental health. The annual global cost of HL is over \$980 billion annually (1). To prevent and treat HL, extensive research has been devoted to investigating its common causes, such as noise exposure, aging, and ototoxic drug use; the role of exposure to environmental heavy metals in HL is especially concerning (2).

Arsenic is a highly toxic heavy metal. Natural sources such as mineral weathering, geothermal processes, and volcanic terrain lead to a massive distribution of arsenic in soil and water. Arsenic is widely used in anthropogenic activities such as mining, fossil fuel combustion, pesticides/herbicides, and wood timber and leather preservation (3, 4). Arsenic is found in different oxidation states and multiple inorganic and organic forms, which can be converted into each other in nature (5). Animals convert inorganic arsenite and arsenate into methylarsonic acid, dimethylarsinic acid, monomethylarsonous acid, and dimethylarsinous acid in a series of methylating reactions (5). Most arsenic is distributed to the kidneys, muscles, liver, nerve tissue, and mainly excreted from the mammalian body in the urine (5). Metabolic inhibition, oxidative stress, genotoxicity, and several epigenetic factors including histone modifications and micro-RNA are believed to be the underlying mechanisms of arsenic toxicity (5). The World Health Organization has recommended the maximum acceptable arsenic concentration in drinking water as 10 µg/L (10 ppb) (5). More than 200 million people worldwide are estimated to be exposed to unsafe levels of arsenic (5), which poses a health risk to humans. Previous studies have shown that human exposure to arsenic is associated with cancer of the kidney, lung, bladder, skin, prostate, and liver (6); cardiovascular disorders (7), neurological diseases, and complications in children (8) and adults (9); and diabetes mellitus (10), reproductive disorders, and developmental disorders (11).

Studies investigating the relationship between arsenic and HL are contradictory and have mostly focused on individuals living or working in arsenic-contaminated areas or those with acute high doses of arsenic poisoning (12–22). Bencko et al. (12) reported low-, and high-frequency HL in 56 10-year-old children residing near a power plant burning local coal of high arsenic content. Another study investigating 145 Bangladeshi living in areas of arsenic-polluted drinking water showed levels of arsenic in toenails were significantly correlated with HL at 4 kHz (13). Shokoohi et al. reported increased risk of HL in 120 cases exposed to arsenic in Iran (14). Sugiyama et al. reported that 70.5% of 190 cases had resided near an arsenic mine had HL, with difficulty to rule out the possibility of other factors, like age which may contribute to HL (15). Li et al. reported 48 cases drinking tube well water contaminated with arsenic had significantly

higher risks of HL at 4 kHz (16). Investigations carried out on 13,021 people drinking arsenic contaminated water (50–1860 µg/L) in Inner Mongolia, China showed 5.88% of HL (17). De Capitani et al. reported a case of severe HL in a young male due to acute poisoning from pesticide monosodium methanarsenate containing organic arsenicals (18). However, Milham et al. (19) investigated the hearing of 7 children with high average urinary arsenic levels, who were living near a copper smelter, and found their hearing were within normal limits. Supapong et al. (20) reported 27 females drinking water from shallow wells which had a low level of arsenic showing no difference in brainstem auditory evoked potentials compared with the referent group. A study of 121 workers exposed to arsenic in Taiwan, China showed no relationship between arsenic exposure and hearing thresholds (21). Another study showed no significant correlation between arsenic concentrations and hearing parameters in 59 artisanal gold miners in Nicaragua whose nail arsenic concentrations were far exceeded reference levels (22). Only two studies have reported the effect of arsenic on hearing in the general population (2, 23). One study showed no overall association between quartiles of urinary arsenic levels and HL in US adolescents (2). Another study showed no evidence of association between urinary arsenic level and hearing disturbance in US adults (23). However, self-reported hearing conditions instead of pure tone audiometry data were used in the analysis. Here, we performed a large-scale cross-sectional study to investigate the relationship between urinary arsenic and hearing levels in adults aged 20–69 years who participated in the United States (US) National Health and Nutrition Examination Survey (NHANES) 2015–2016, which is the most recent survey cycle.

2 Methods

2.1 Study design and population

The NHANES is a nationwide cross-sectional survey conducted by the US Center for Disease Control and Prevention that collects health-related representative data of US non-institutional civilians on a 2-year basis; this consists of in-person interviews, physical examinations, and laboratory test results using a stratified multistage probability sampling design. All NHANES data analyzed in this study are publicly available on the NHANES official website.¹ The National Center for Health Statistics Research Ethics Review Board approved the NHANES, and all participants provided written informed consent.

Participants in the NHANES cycle 2015–2016, which contained test data for both urinary arsenic metabolites and audiometric examinations of adults aged 20–69 years were included in this study. A total of 1,017 individuals were included in the final analysis. The participants with abnormal otoscopic examination results, poor-quality tympanogram results or tympanogram of Type B or Type C were excluded to avoid analyzing conductive or mixed HL data. Figure 1 illustrates the selection procedure used in this study.

Abbreviations: BMI, body mass index; HL, hearing loss; NHANES, National Health and Nutrition Examination Survey; PTA, pure-tone average; uAs, urinary arsenic; uDMA, urinary dimethylarsinic acid.

1 <https://www.cdc.gov/nchs/nhanes/continuousnhanes/default.aspx?BeginYear=2015>

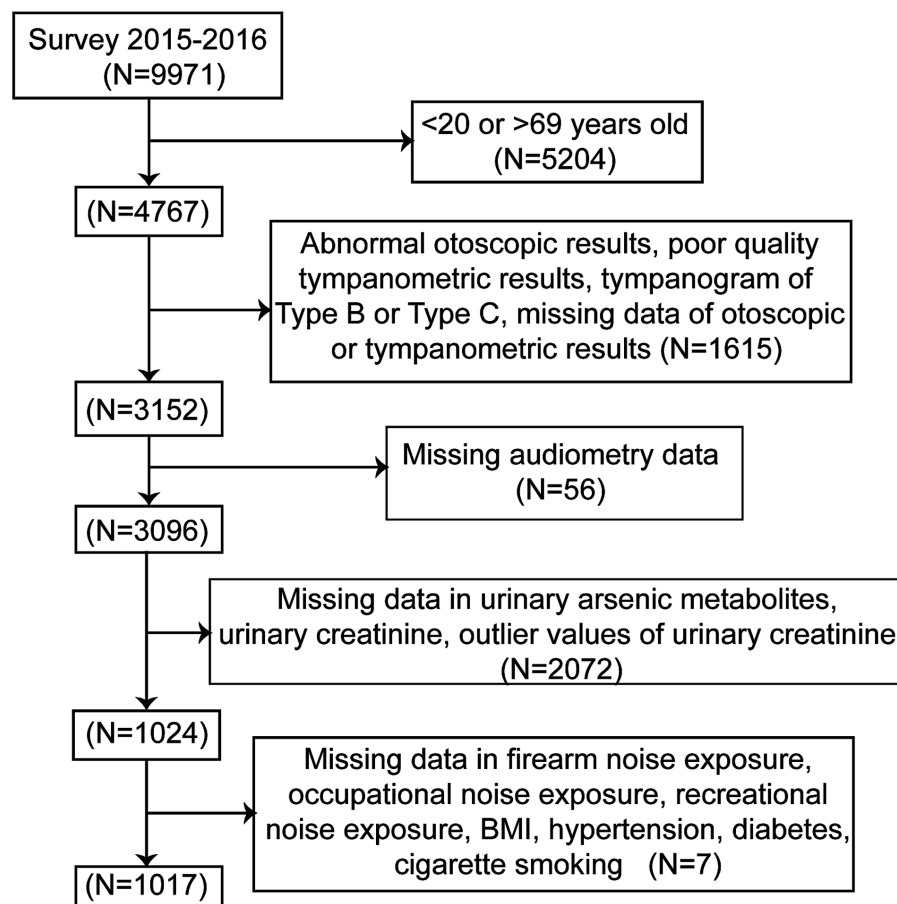


FIGURE 1

Flow chart of the selection process. NHANES, National Health and Nutrition Examination Survey.

2.2 Urinary arsenic measures

Urine is analyzed because arsenic is mainly excreted from the mammalian body in the urine. Urine samples were collected from all examined participants aged 3–5 years and those aged ≥ 6 years from a one-third subsample. Vials holding urine samples were stored under appropriate frozen (-30°C) conditions until shipped to National Center for Environmental Health, Center for Disease Control and Prevention for testing. Concentrations of arsenic metabolites were measured using inductively coupled-plasma dynamic reaction cell-mass spectrometry (ICP-DRC-MS), which can achieve rapid and accurate quantification of total urinary arsenic. The NHANES quality assurance and quality control (QA/QC) protocols meet the 1988 Clinical Laboratory Improvement Act mandates. Several techniques were used to monitor laboratory team performance. Details are available on the NHANES official website.² Arsenic metabolites with a detection rate $\geq 60\%$ in the samples were selected for analysis in our study including total urinary arsenic (uAs) and urinary dimethylarsinic acid (uDMA). Other arsenic compounds with a detection rate of $<60\%$ in the urine, such as arsenous acid, arsenic acid, arsenobetaine, arsenocholine, and monomethylarsonic acid, were excluded. The limit of detection divided by the square root of two was

used for any sample below the limit of detection. The uAs concentrations were ln-transformed to normalize the skewed distribution before the analysis (23, 24). The ln-transformed creatinine-adjusted uAs concentrations were not used because the data were skewed.

2.3 Audiometric measurement

Audiometric measurements were performed by trained examiners at the mobile examination center. Participants aged 20–69 years were included in the study. An otoscopic examination of the ear canals and eardrums was conducted for excessive ear cerumen and other physical abnormalities. Tympanometry was performed using a tympanometer (Interacoustics Model Titan, Denmark to identify middle ear pathologies that may contribute to conductive HL). An audiometer (Interacoustics Model AD226, Denmark) was used to measure the pure-tone air-conduction audiometry of both ears. Participants who were unable to remove their hearing aids or could not tolerate the headphone-induced ear pain were excluded. The complete detailed procedure is available on the website.³

2 https://wwwn.cdc.gov/Nchs/Nhanes/2015-2016/UTAS_I.htm

3 https://wwwn.cdc.gov/Nchs/Nhanes/2015-2016/AUX_I.htm

In this study, the low-frequency pure-tone average (PTA) was defined as a PTA of 0.5, 1, and 2 kHz; speech-frequency PTA as a PTA of 0.5, 1, 2, and 4 kHz; and high-frequency PTA as a PTA of 4, 6, and 8 kHz in the better-hearing ear. The World Health Organization (WHO) criteria 2021 defined HL as speech-frequency PTA ≥ 20 dB in the better-hearing ear (1).

2.4 Potential covariates

Age, sex, race, educational level, body mass index (BMI), hypertension, diabetes, cigarette smoking, firearm noise exposure, occupational noise exposure, and recreational noise exposure were included as potential covariates. Information on demographic and potential hearing-related covariates, including age, sex, race, educational level, cigarette smoking, firearm noise exposure, occupational noise exposure, and recreational noise exposure, was obtained through home-based interviews. BMI was calculated from the weight and height data of the participants, which were obtained during physical examinations at a Mobile Examination Center.

Self-reported hypertension was defined as a “Yes” response to the question, “Have you ever been told by a doctor or other health professional that you had hypertension, also called high blood pressure?” or participant-reported treatment for high blood pressure. Hypertension was defined as a mean blood pressure (from three measurements) ≥ 130 mm Hg for systolic blood pressure or ≥ 80 mm Hg for diastolic blood pressure (25). Participants were considered to have diabetes if they met any of the following conditions: fasting plasma glucose ≥ 126 mg/dL, oral glucose tolerance test ≥ 200 mg/dL, glycated hemoglobin $\geq 6.5\%$, self-reported diabetes, and currently on insulin or oral medication use (26). Participants were categorized as never, former, or current smokers from the question, “Have you smoked at least 100 cigarettes in your entire life?” and “Do you now smoke cigarettes?” (27). Firearm noise exposure was defined as “ever used firearm for any reason” (27). Occupational noise exposure was defined as “ever had a job exposure to loud noise for ≥ 4 h a day, several days a week” (27). Recreational noise exposure

was defined as “outside of a job, ever been exposed to very loud noise or music for ≥ 10 h a week” (27).

2.5 Statistical analysis

This study used subsample A weights (WTSA2YR) of 2015–2016 NHANES cycle to estimate representative measures for the civilian non-institutionalized US population, following the analytic guidelines of the National Center for Health Statistics (28). uAs and uDMA levels were Ln-transformed and classified into tertiles. The weighted demographic characteristics of the study participants grouped by the tertiles of uAs were evaluated. Data are reported as numbers and percentages for categorical variables or as mean $\pm$ standard deviation (SD) for continuous variables. Regression coefficients (β) and 95% confidence intervals (CIs) were generated using multivariate linear regression models to test the associations between uDMA, uAs and hearing threshold shifts, adjusting for potential covariables, including age, sex, race, education level, BMI, hypertension, diabetes, cigarette smoking status, and noise exposure. Ln-transformed uDMA and uAs levels were converted from continuous variables to categorical variables (tertiles), and the tertiles were modeled as continuous variables to test the linear trend. The interactions of age and sex with uDMA and uAs in influencing hearing thresholds were also tested. Multivariate analyses stratified according to age and sex were performed.

Smoothed curves were fitted using R to explore the relationship between uDMA, uAs, and hearing threshold shifts after adjusting for potential covariates (Figure 2). A weighted two-piecewise linear regression model with a single inflection point was used to examine the effect of uDMA and uAs on hearing threshold shifts, shown as a smoothing plot. A significant log-likelihood ratio test was performed to determine the presence of a threshold. The inflection point was calculated using a recursive algorithm and a maximum-likelihood model. All statistical tests were conducted using R 3.6.1 and R 4.3.1. Statistical p value was set at $p < 0.05$.

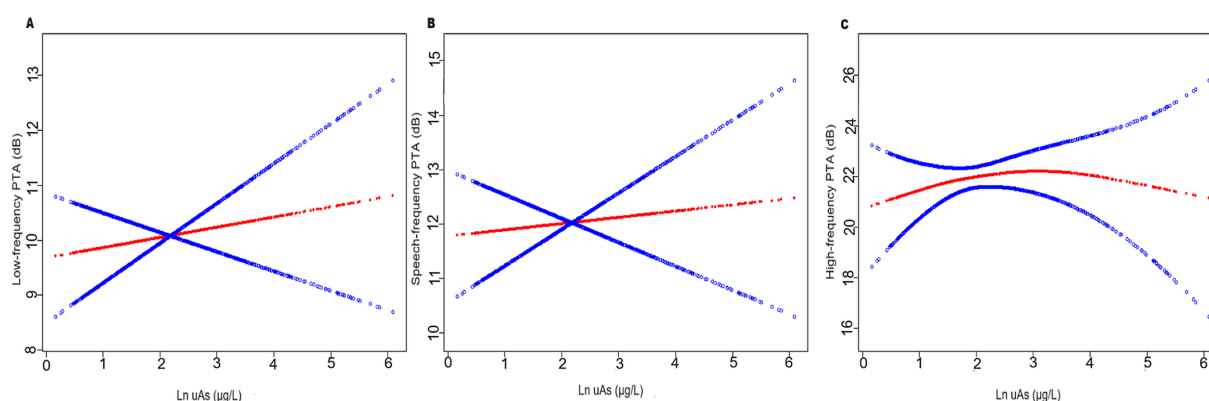


FIGURE 2

The relationship between uAs levels and hearing threshold shifts ($N = 1,017$): (A) low-frequency pure-tone average (PTA), (B) speech-frequency PTA and (C) high-frequency PTA. The red line represents the smooth curve fit between variables. In comparison, blue bands represent the 95% CI. Age, gender, race, education level, BMI, hypertension, diabetes, cigarette smoking, firearm noise exposure, occupational noise exposure and recreational noise exposure were adjusted.

3 Results

3.1 Baseline characteristics

The characteristics of participants according to uAs tertiles are shown in Table 1, and a total of 1,017 participants aged 20–69 years who had measured data for hearing and urinary arsenic levels were included in this study. 45.77% of the participants were men, and the mean age was 42.13 ± 13.66 years old. Overall, 18.43% of adults had sensorineural HL. The ranges of uAs tertiles 1–3 were < 4.17 , $4.17–10.72$ and > 10.72 $\mu\text{g/L}$, respectively. Compared with the lowest tertile, participants in the tertile 3 group tend to had poorer speech- and high-frequency hearing levels, non-Hispanic black ethnicity and other races (all $p < 0.05$).

3.2 The association of arsenic metabolites with hearing levels

We have used two multivariate linear regression models to show the relationship between arsenic metabolites uDMA and uAs levels with low-, speech-, and high-frequency hearing levels in Table 2: crude model, no covariate was adjusted; adjusted model, age,

gender, race, education level, BMI, hypertension, diabetes, cigarette smoking, firearm noise exposure, occupational noise exposure, recreational noise exposure were adjusted. We found a significantly positive association between uDMA levels and low-, and speech-frequency PTA hearing threshold shifts in both the unadjusted model (crude model) and adjusted model. Individuals in the tertile 3 group (uDMA > 5.63 $\mu\text{g/L}$) had worse low-, and speech-frequency PTAs compared with those in the tertile 1 group (uDMA < 2.22 $\mu\text{g/L}$). Regarding uAs, although we found a significantly positive association between uAs levels with hearing levels of all frequencies in the crude model, no significant associations were found for the adjusted model.

Supplementary Tables S1, S2 showed no significant association of both uDMA and uAs levels with hearing levels in the subgroup analysis stratified by age and sex, respectively.

3.3 Analyses of the non-linear relationship between arsenic metabolites and hearing levels

The relationship between uAs and high-frequency PTA was found to be nonlinear rather than linear (P for nonlinearity = 0.014, Table 3). The non-linearity relationship between uAs levels and high-frequency PTA

TABLE 1 The weighted demographic characteristics of study participants by uAs.

Variables	Total	Tertile 1 (N = 339)	Tertile 2 (N = 339)	Tertile 3 (N = 339)	P^a
Age, years	42.13 ± 13.66	41.23 ± 13.84	42.20 ± 12.64	43.27 ± 13.33	0.1526
Low-frequency PTA, dB ^b	9.94 ± 10.20	9.29 ± 10.98	9.78 ± 8.98	11.03 ± 10.26	0.0822
Speech-frequency PTA, dB ^b	11.97 ± 11.37	11.04 ± 11.83	11.86 ± 10.84	13.39 ± 11.16	0.0266
High-frequency PTA, dB ^b	22.28 ± 18.82	20.91 ± 17.68	21.94 ± 20.20	24.57 ± 18.52	0.0380
BMI, kg/m ²	29.69 ± 7.21	29.51 ± 7.39	29.98 ± 7.09	29.62 ± 7.08	0.6771
Male, %	45.77	42.11	50.01	46.08	0.1032
Race, %					<0.0001
Mexican American	9.83	8.58	13.49	7.46	
Non-Hispanic White	62.11	73.58	56.65	52.34	
Non-Hispanic Black	11.37	7.22	12.26	16.12	
Other races	16.69	10.62	17.60	24.09	
Education level, %					0.4594
Below high school	12.82	11.15	12.36	15.68	
High school	18.31	19.01	17.29	18.49	
Above high school	68.86	69.84	70.35	65.83	
Hypertension, %	40.54	37.36	44.56	40.43	0.1439
Diabetes, %	13.29	10.82	14.01	15.89	0.1376
Cigarette smoking, %					0.7388
Never smoker	58.13	59.14	59.37	55.31	
Former smoker	24.23	22.67	24.41	26.19	
Current smoker	17.65	18.19	16.22	18.51	
Firearm noise, %	52.92	56.31	50.87	50.53	0.2166
Occupational noise, %	29.99	30.81	30.83	27.92	0.6607
Recreational noise, %	14.05	14.15	14.34	13.59	0.9630
°Hearing loss, %	18.43	15.99	16.97	23.44	0.0321

NHANES 2015–2016 cycle, $N = 1,017$. PTA pure-tone average, BMI body mass index.

^a p values of continuous variables and categorical variables were calculated by weighted linear regression model and weighted chi-square test, respectively.

^bLow-, speech-, high-frequency PTA values in the better ear were computed from the average of hearing thresholds of 0.5, 1 and 2 kHz, 0.5, 1, 2 and 4 kHz, 4, 6 and 8 kHz, respectively.

°Earing loss was defined as the PTA value ≥ 20 dB in the better ear.

Significant values are in bold.

TABLE 2 Multivariable linear regression models assessing the relationship between uDMA, uAs levels and PTA hearing thresholds (N = 1,017).

		Ln uDMA (µg/L)			P _{trend}
		Tertile 1 (N = 339)	Tertile 2 (N = 337)	Tertile 3 (N = 341)	
Low-frequency PTA	Crude βs	Ref	0.49 (−0.98, 1.96)	2.42 (0.86, 3.97)	0.0033
	Adjusted βs	Ref	−0.12 (−1.47, 1.23)	2.29 (0.83, 3.74)	0.0040
Speech-frequency PTA	Crude βs	Ref	1.02 (−0.62, 2.66)	2.73 (0.99, 4.46)	0.0023
	Adjusted βs	Ref	−0.07 (−1.46, 1.33)	2.38 (0.88, 3.88)	0.0036
High-frequency PTA	Crude βs	Ref	1.09 (−1.63, 3.81)	2.30 (−0.58, 5.19)	0.1163
	Adjusted βs	Ref	−1.35 (−3.36, 0.65)	1.41 (−0.74, 3.57)	0.2920

		Ln uAs (µg/L)			P _{trend}
		Tertile 1 (N = 339)	Tertile 2 (N = 339)	Tertile 3 (N = 339)	
Low-frequency PTA	Crude βs	Ref	0.49 (−1.00, 1.98)	1.74 (0.20, 3.28)	0.0302
	Adjusted βs	Ref	0.18 (−1.19, 1.55)	1.08 (−0.36, 2.51)	0.1528
Speech-frequency PTA	Crude βs	Ref	0.83 (−0.83, 2.48)	2.35 (0.64, 4.07)	0.0079
	Adjusted βs	Ref	0.25 (−1.17, 1.66)	1.41 (−0.07, 2.89)	0.0704
High-frequency PTA	Crude βs	Ref	1.03 (−1.71, 3.77)	3.66 (0.82, 6.50)	0.0132
	Adjusted βs	Ref	−0.18 (−2.21, 1.85)	1.68 (−0.44, 3.80)	0.1432

Crude Model = unadjusted. Adjusted Model = Crude Model + age, gender, race, education level, BMI, hypertension, diabetes, cigarette smoking, firearm noise exposure, occupational noise exposure, recreational noise exposure; PTA, pure tone average; CI, confidence interval. Significant values are in bold.

indicated an inverted U-shaped pattern (Figure 2). Using a two-part piecewise linear regression model with all confounders being adjusted, the high-frequency PTA increased with increasing uAs levels until the turning point of 1.54, whereas it did not as uAs exceeding 1.54 ($\beta = 4.53$; 95% CI: 1.16, 7.90; $p = 0.0085$) (Table 3). No nonlinear relationship was observed between uDMA and hearing threshold shifts (Supplementary Tables S3).

4 Discussion

In this nationwide cross-sectional study, we explored, for the first time, the relationship between uAs levels and low-, speech-, and high-frequency hearing threshold shifts among adults aged 20–69 years, using the NHANES 2015–2016 database.

Our study found a significant linear association between uDMA levels and poor low-, and speech-frequency PTAs, with no significant interactions of uDMA level with age or sex. Additionally, a significant non-linear association was observed between the uAs levels and poor high-frequency PTA in adults in the United States. These results are consistent with those of previous studies (12, 13, 15–18). Bencko et al. reported that arsenic-exposed children living near a power plant that burned high arsenic-containing coal in Czechoslovakia had significantly worse pure-tone thresholds than non-exposed children at 125, 250, and 8,000 Hz (low and high frequencies) (12). Guo et al. reported a higher prevalence of hearing impairment in adults in counties with drinking water contaminated with naturally occurring arsenic in inner Mongolia, China (17). Xiang et al. reported that individuals aged 12–29 years who had been drinking arsenic-contaminated tube well water in Bangladesh had a significantly higher risk of hearing impairment at 4 kHz than those in the control group, showing that oral exposure to arsenic harms hearing in young adults (16). The same research group also found that the levels of arsenic in toenails, but not in urine, were significantly correlated with HL at 4, 8, and 12 kHz in 145 Bangladeshi individuals aged 12–55 years (13). A descriptive study by Sugiyama et al. reported that 70.5% of

residents near an arsenic mine in Toroku, Japan, experienced deafness 45 years after the initial arsenic exposure (15). A case report by De Capitani et al. showed a severe bilateral neurosensory pattern of deafness with symmetrical losses of 110–120 dB at all tested frequencies in both ears of a 36-year-old man who had attempted suicide by ingesting approximately 250 mL of monosodium methanarsenate, a pesticide containing synthetic forms of organic arsenicals (18). Auditory brainstem-evoked potentials were absent bilaterally after maximum stimulation (105 dB), confirming severe cochlear lesions in both ears (18).

In addition to investigations concerning hearing impairment in individuals living or working in arsenic-polluted areas or those with acute arsenic poisoning, the results of our large-scale research imply a harmful effect of environmental arsenic exposure on the hearing system in the general population in the US. In many countries worldwide, including the US, India, Bangladesh, China, and Mexico, a high level of inorganic arsenic is naturally present in groundwater (29). Almost half of the world's population uses groundwater for domestic needs (30). In addition to groundwater, arsenic is found in common dietary sources including rice, grains, juices, and seafood (5, 31). uDMA, an inorganic arsenic, and total arsenic uAs were detected in more than 60% of samples. Arsenic can impair low- and high-frequency hearing in individuals without them being aware because of its relatively low extent and lack of influence on speech-fluency PTA (23). The harmful effects of arsenic on human hearing are concerning. The results of our study were contrary to those of previous studies that showed no relationship between arsenic levels and human hearing (2, 14, 19–23). Variations in the sample size, study design, analytical methodology, control for different covariates, and racial heterogeneity may explain the disparities between our results and those of previous studies.

Although several human-based association studies have revealed a link between arsenic exposure and hearing status, the mechanistic impact of arsenic exposure on the underlying auditory pathogenesis remains poorly understood. An animal experiment showed that mice exposed to arsenic ex vivo exhibited significantly decreased numbers

TABLE 3 The results of two-piecewise linear regression model between uAs levels and hearing thresholds (N = 1,017).

Exposure variables	Low-frequency PTA	Speech-frequency PTA	High-frequency PTA
Cut off point of uAs	3.86	2.56	1.54
< Cut off point of uAs	0.76 (0.04, 1.48), 0.0380	1.36 (0.31, 2.41), 0.0110	4.53 (1.16, 7.90), 0.0085
≥ Cut off point of uAs	−1.03 (−3.85, 1.79), 0.4752	−0.56 (−1.82, 0.70), 0.3866	−0.43 (−1.57, 0.71), 0.4600
Loglikelihood ratio test	0.263	0.052	0.014

Significant values are in bold.

of auditory neurons and fibers (16). Other experiments observed extravasates in different parts of the middle and inner ear, inner ear cavities filled with a serous or serofibrinous transudate, and degeneration of the cells in the spiral and vestibular ganglion (32). Oxidative stress, metabolic inhibition, genotoxicity, and epigenetic alterations, including micro-RNA-dependent regulation, are some of the molecular mechanisms underlying arsenic toxicity (5).

This study was conducted using large-scale population-based representative data with adjustments for critical confounders and rigorous quality control during data collection to establish a relationship between urinary arsenic concentrations and hearing impairment. However, this study had a few limitations. First, interpretation of our findings is strongly limited by the cross-sectional design of this study. The temporality of the association between exposure and outcomes could not be confirmed, so causation could not be established from our study. As with all cross-sectional human studies, residual confounding and confounding by unmeasured variables cannot rule out. There are some potential confounders we could have wished to control but were unable to calculate in our study, like dietary intake of all the ototoxic drugs, history of ear infection, congenital hearing impairment, chlorinated pollutants, et al. Prospective and Mendelian randomization studies are required to demonstrate the association between arsenic exposure and hearing levels definitively. Second, only urine sample data were used in the analysis. Nail and blood sample data can be compared to determine which is more sensitive in reflecting the relationship between arsenic exposure and hearing levels. Third, the data pertained only to US adults; caution should be exercised when generalizing the findings to people from other countries and races. Furthermore, additional basic studies are required to elucidate the effects of arsenic on auditory pathogenesis.

5 Conclusion

The results of the NHANES data analyses revealed a significant linear association between uDMA levels and poor low-, and speech-frequency PTAs, with no difference among participants by age or sex. Additionally, a significant non-linear association was observed between uAs levels and poor high-frequency PTA in US adults. Exposure to arsenic in the general population may harm the hearing system. Further research is essential to verify our results given the cross-sectional nature of the NHANES.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The studies involving humans were approved by the National Center for Health Statistics Research Ethics Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

LL: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. ZJ: Resources, Supervision, Validation, Writing – review & editing. TL: Resources, Supervision, Validation, Writing – review & editing.

Funding

The authors declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

All authors would like to thank NHANES for providing the publicly available data.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpubh.2024.1431122/full#supplementary-material>

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OPEN ACCESS

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RECEIVED 23 November 2024

ACCEPTED 16 December 2024

PUBLISHED 06 January 2025

CITATION

Chen Z, Qu H, Sun J, Wang T, Yuan Y, Gu J, Bian J, Liu Z and Zou H (2025) CPT1 deficiency blocks autophagic flux to promote lipid accumulation induced by co-exposure to polystyrene microplastic and cadmium. *Front. Pharmacol.* 15:1533188. doi: 10.3389/fphar.2024.1533188

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CPT1 deficiency blocks autophagic flux to promote lipid accumulation induced by co-exposure to polystyrene microplastic and cadmium

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Introduction: Cadmium (Cd) and polystyrene microplastics (PS-MPs), two ubiquitous environmental contaminants, produce unique synergistic toxicity when co-existing. Key unanswered questions include specific effects on liver function and potential mechanisms.

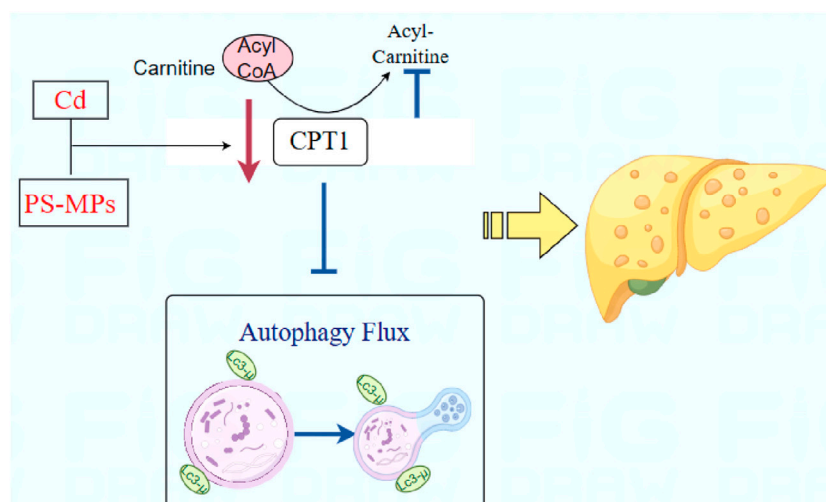
Methods: In this study, C57BL/6 mice and AML12 cells were used to establish *in vivo* and *in vitro* models to elucidate the effects of combined exposure to PS-MPs and Cd on the liver and their mechanisms.

Results: The results showed that the combined effects of PS-MPs and Cd caused significantly more liver damage than exposure alone. As observed by transmission electron microscopy (TEM), the number of autophagosomes was significantly increased in the PS-MPs and Cd co-treated group. In addition, autophagic flux was assayed by RFP-GFP-LC3, a reporter system expressing dual fluorescent proteins, which showed an overwhelming enhancement of autophagic flux damage by co-exposure to PS-MPs and Cd compared to exposure alone. To further investigate the involvement of carnitine palmitoyltransferase1 (CPT1) in liver injury induced by co-exposure to Cd and PS-MPs, we co-exposed Baicalin, an activator of CPT1, with PS-MPs and Cd, and showed that activation of CPT1 alleviated the impairment of autophagic fluxes induced by co-exposure of Cd and PS-MPs and further alleviated the changes in lipid accumulation and associated protein levels.

Discussion: In conclusion, the concurrent exposure of PS-MPs and Cd resulted in the blockage of hepatic lipid accumulation and autophagic pathway and further aggravated the toxic damage to the liver. Activation of CPT1 could alleviate the PS-MPs and Cd-induced lipid accumulation and autophagy pathway blockage thus reducing liver injury.

KEYWORDS

autophagy, cadmium, liver, lipid metabolism, polystyrene microplastics



GRAPHICAL ABSTRACT

Highlights

1. PS-MPs accumulate in mouse liver and promote Cd accumulation.
2. Co-exposure to PS-MPs and Cd exacerbate liver injury caused by exposure alone.
3. Co-exposure to PS-MPs and Cd exacerbates lipid accumulation and blockage of autophagic flux.
4. Activation of CPT1 attenuates the blockage of autophagic flux induced by co-exposure of PS-MPs and Cd, thereby reducing lipid accumulation.

1 Introduction

Cadmium (Cd) is a heavy metal that is ubiquitous in the environment and originates mainly from human agricultural production and industrial activities (Genchi et al., 2020). Cd is produced about 13,000 tonnes per year worldwide and is the most toxic pollutant in industrialized countries (Mężyńska et al., 2018). Currently, Cd pollution has become an unavoidable problem in the ecosystem (Zhu et al., 2018). The United Nations included Cd and its compounds in the list of carcinogens in 2017. Several studies have shown an association between Cd exposure and obesity, diabetes, and metabolic syndrome, as well as an increased risk of atherosclerosis and cardiovascular disease (Fagerberg and Barregard, 2021; Tinkov et al., 2017). In the body, the liver is the main site of Cd accumulation (Mężyńska and Brzóska, 2019). Studies have shown that Cd exposure is positively associated with the incidence of many liver diseases, including necrotizing inflammation, hyperglycemia, non-alcoholic fatty liver disease (NAFLD), and non-alcoholic steatohepatitis (NASH) (Rosales-Cruz et al., 2018). Mitochondrial β -oxidation of fatty acids is a vital pathway for fatty acid catabolism, which plays an indispensable role in regulating energy homeostasis across the entire body. CPT1 is the essential enzyme in carnitine-dependent transport across the inner mitochondrial membrane. The reduction in its quantity leads

to a lower rate of beta-oxidation of fatty acid (Schlaepfer and Joshi, 2020).

Microplastics (MPs) are plastic fragments or particles less than or equal to 5 mm in diameter found in the environment. Polystyrene is a highly productive plastic and is among the most commonly occurring microplastics (MPs) in the environment (Vaid et al., 2021). It can be transported to more places by wind, snow, atmosphere, and various water currents (rivers, lakes, and oceans) (Xiang et al., 2022; Chen et al., 2020; Evangelidou et al., 2020; Luo et al., 2019a; Abbasi et al., 2019). After entering the organism, most of the MPs are excreted in the feces, especially large particles with a diameter greater than 150 μ m, and smaller particles accumulate in certain organs of the body, such as the brain, heart, liver, kidneys, and epididymis (Liang et al., 2021). Current studies have shown that MPs ranging from 25 nm to 90 μ m can accumulate in the liver of marine fish or mammals (rats and mice) (Babaei et al., 2022; Ding et al., 2020; Pitt et al., 2018) and are negatively correlated with MPs size (Abarghouei et al., 2021). Studies have shown that exposure of zebrafish to PS-MPs for 3 weeks resulted in hepatocellular necrosis, inflammatory infiltration, and lipid accumulation (Lu et al., 2016). Vacuolar degeneration and inflammatory infiltration were observed in mouse liver after 30 days of exposure to PS-MPs (Luo et al., 2019b). Currently, the main body of research on liver damage caused by MPs exposure is mostly marine organisms, and more experiments are needed to provide theoretical support for mammalian studies (Yin et al., 2022).

Both MPs and Cd are prevalent pollutants in the environment, and human activities have caused large amounts of Cd and MPs to enter water streams and soils, be ingested by aquatic organisms and plants, and accumulate through the food chain (Allen et al., 2021; Weber et al., 2022; Shi et al., 2016). MPs can serve as effective carriers of other pollutants in the surrounding environment, adsorbing organic pesticides, toxic heavy metals, and atmospheric pollutants (Martinho et al., 2022). Relevant researchers have detected the presence of different concentrations of heavy metals in MPs in the environment (Holmes et al., 2012). It has also been reported in the literature that adsorbed heavy metals were found in plastics in flying

birds (Massos and Turner, 2017). An acute toxicity study of PS-MPs with Cd on grass carp showed that the presence of PS-MPs increased the concentration of Cd in grass carp and accelerated their death (Chen et al., 2022). A chronic toxicity experiment in zebrafish exposed to PS-MPs and Cd was consistent with its results, showing that the presence of MPs did enhance the toxicity of Cd (Lu et al., 2018a). While the effects of co-exposure of Cd and PS-MPs on the mouse liver and its mechanism are still unclear. Therefore, in this study, we used C57BL/6 mouse and AML12 cells, treated mice and cells with PS-MPs and Cd individually or in combination, explored whether PS-MPs could exacerbate Cd-induced liver damage. This study will reveal the toxicity mechanism of Cd and PS-MPs on the liver and provide a theoretical basis for the targeted prevention and treatment of Cd and PS-MPs-induced liver injury.

2 Materials and methods

2.1 Chemicals and antibodies

StubRFP-sensGFP-LC3 Lentivirus was purchased from Genechem (Shanghai, China). Cadmium chloride (CdCl_2) was purchased from Sigma-Aldrich (St. Louis, MO, United States). Polystyrene microspheres (PS-MPs) (5 μm) (2.5%w/v, 10 mL) was purchased from BaseLine ChromTech Research Centre (Tianjin, China). Baicalin was purchased from MedChemExpress (Shanghai, China). Hematoxylin and Eosin Staining Kit, Oil Red O Staining Kit, and Hoechst 33342 were purchased from Beyotime Biotechnology (Shanghai, China). BODIPY[™] 493/503 was purchased from ThermoFisher Scientific (Waltham, MA, United States). ACC, CPT1, Atg7, β -actin, Beclin1, P62, and LC3 monoclonal antibody were purchased from Cell Signaling Technology (Danvers, MA, United States). Apob and Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G were purchased from Santa Cruz Biotechnology (CA, United States). Triglyceride (TG) assay kit, Total cholesterol (T-CHO) assay kit, and High-density lipoprotein cholesterol (HDL-C) assay kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2 Experimental design

In accordance with the Yangzhou University Animal Use Protocol [IDS: SYXK (Su) 2017-0044]. This study followed previously established methods (Liang et al., 2021; Lu et al., 2016; Zhang et al., 2018), using PS-MPs obtained from BaseLine ChromTech Research Centre (Tianjin, China). Water is the main source of PS-MPs and Cd pollution, so this study is based on the concentration of Cd and PS-MPs pollution in the environment and combined with experimental requirements and previous studies. The concentrations of Cd (50 mg/L) and PS-MPs (10 mg/L) were selected (Ma et al., 2021; Prokić et al., 2021; Sun et al., 2021; Yin et al., 2023). C57BL/6 mice (6 weeks old, SPF grade) obtained from Spelford Biotechnology (Beijing, China) underwent a 1-week acclimation period. The 32 mice were randomly divided into four groups: 1) The control group [receiving double distilled water (DDW)]; 2) the PS-MPs group (administered with DDW containing 10 mg/L PS-MPs

with a particle size of 5 μm); 3) the Cd group (administered with 50 mg/L Cd), and 4) the PS-MPs and Cd co-treatment group (Cd + PS-MPs, administered with purified water containing 50 mg/L Cd and 10 mg/L PS-MPs with a particle size of 5 μm). Mice were given free access to water for 3 months, during which time food intake was uncontrolled. At sacrifice by cervical dislocation, body weight was measured before the liver was removed for fixation in 10% neutral formalin or 2.5% glutaraldehyde. The relevant experiments in this study refer to previously published content (Sun et al., 2023a; Sun et al., 2023b; Wang et al., 2023) and manufacturer's instructions.

2.3 Observation of the morphology of PS-MPs by scanning electron microscopy

Transfer 1 mL of PS-MPs solution to a 1.5 mL EP tube and centrifuge for 20 min at 12,000*g at 4°C. To acquire PS-MPs precipitation, dispose of the supernatant. The EP tube should then be placed in an oven at 60°C to completely evaporate the water and obtain PS-MPs powder. PS-MPs will be observed via SEM.

2.4 Laser confocal microscopy characterizes distribution of PS-MPs

Two male C57BL/6 mice were administered fluorescent PS-MPs via gavage individually for a duration of 1 week. After the treatment period, the mice were euthanized, and the livers were extracted to prepare frozen sections. The nuclei within the samples were stained with Hoechst 33342. Observation of the slices was done using a laser confocal microscope.

The cells were then cultured under standard conditions until the cell density reached 80%. The initial culture medium was discarded and substituted with fluorescent PS-MPs medium, which was prepared with serum-free medium. The samples were then processed for 12 h. The samples were rinsed twice with PBS and fixed using 4% paraformaldehyde for 20 min at room temperature. Following the rinse with PBS, they were incubated in Hoechst 33342 under light protection for 20 min. Finally, observation of the slices was done using a laser confocal microscope.

2.5 Hematoxylin and eosin staining

The samples were retained in paraformaldehyde for 24 h and subsequently trimmed. The fixed tissues were embedded in wax blocks to create paraffin sections. These sections were then dehydrated in a graded manner and stained with H&E before being washed with running tap water. The observations were made using a microscope manufactured by Leica Corporation (model number: Leica 2500) located in Wetzlar, Germany.

2.6 Transmission electron microscope (TEM)

Fresh liver tissue from each group was sliced into segments measuring 1–2 mm. These segments were then treated with 2.5% glutaraldehyde fixative for overnight fixation at a temperature of

4°C. Following fixation, the segments were subjected to osmic acid fixation, underwent dehydration through a graded ethanol series, and were finally embedded in the Epon 812 compound. A diamond knife was employed to cut ultrathin sections, which were negatively stained using aqueous uranyl acetate and lead citrate.

2.7 Cell culture and morphological observations

The AML12 cell line (American Type Culture Collection) was grown in Dulbecco's modified Eagle's medium (DMEM) with F-12 and 1% ITS-A (Insulin-Transferrin-Selenium-Sodium Pyruvate) (Sigma-Aldrich, St. Louis, MO, United States), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin. The cells were maintained at 37°C under a 5% CO₂ atmosphere. Upon achieving an initial cell density of approximately 80%, they were allocated to four groups: control group, Cd (5 µM) group, PS-MPs (10 µg/mL) group, or Cd + PS-MPs group. Cells underwent 12 h of treatment with defined media prepared from serum-free media post-discarding the existing medium. Post-washings with PBS, images were captured via microscopy observation.

2.8 TG, TC and HDL-C content determination

This experiment was performed according to the reagent manufacturer's instructions. Tissue specimens were weighed, and then mixed with normal saline at a ratio of 1:9. The mixture was homogenised and centrifuged at 2500*g for 10 min before the supernatant was collected for measurement. Similarly, cell specimens were suspended in normal saline, disrupted using ultrasound, centrifuged and the supernatant was then collected for assessment. The TG, TC and HDL-C levels in the supernatants were subsequently analysed using the relevant test kits.

2.9 Cellular index assay

Initially, 100 µL of media was used to establish a baseline in each well. Following this stage, 100 µL of cell suspension (containing an appropriate amount of cells) was added per well, vortexed, and introduced into our detection apparatus. The cells were left undisturbed for a period of 15 min before the start of Cellular index (CI) monitoring, which occurred automatically every 15 min. Once the cell density exceeded 80%, subsequent treatments were initiated while maintaining CI monitoring and normalization.

2.10 Oil red O staining

This experiment was performed according to the reagent manufacturer's instructions. Cells were seeded into 6-well plates and treated when cell density reached around 80%. The initial medium was removed, and the necessary concentration of culture medium pre-prepared with serum-free medium was added for 12 h. After removing the medium, PBS was used to rinse the wells once,

and 1 mL of staining wash solution was added to each one and left for 20S. The wash solution was removed, and each well was treated with 1 mL of modified oil red O staining solution for 30 min. After completing the staining process, the wash solution was added and left for 30S followed by its disposal. Subsequently, the wash solution was added again and left for 20S. The cells were then washed using PBS for a duration of 20S. Next, PBS was added to each well, ensuring even coverage of the cells. Finally, the samples were placed under a microscope for observation and photography.

2.11 BODIPY staining

This experiment was performed according to the reagent manufacturer's instructions. The samples were prepared in 24-well plates and maintained under standard conditions. Once the cellular density reached 80%, treatments were initiated. The initial media was removed and replaced with pre-prepared culture medium devoid of serum, and left for 12 h. The cells were washed twice with PBS, fixed with 4% paraformaldehyde for 20 min. Next, each well was washed with PBS once before introducing BODIPY 493/503 dye dilution. The dye was added at a volume of 400 µL per well, followed by incubation at 37°C, shielded from light, for 15 min. Subsequently, a PBS wash was done followed by a BODIPY stain rinse, both at room temperature. Another PBS wash was performed immediately before proceeding with Hoechst 33342 incubation for 20 min, also at room temperature. After another wash in PBS, the sections, which had been stained, were sealed for analysis using confocal microscopy.

2.12 Western blotting analysis

Tissue suspensions were homogenised and transferred to a 1.5 mL centrifuge tube. Subsequently, the tube was subjected to centrifugation at 1000*g for 10 min resulting in the initial supernatant. This supernatant was then further centrifuged at 12,000*g for 15 min, yielding the final supernatant. Concerning cell samples, cells were harvested from dishes and were lysed with RIPA buffer for 30 min at 4°C. Further, ultrasonification was performed, and the sample was subjected to centrifugation at 12,000*g for 15 min. Protein concentrations were determined through Bicinchoninic acid assays (YeasenBio Technology, Shanghai, China). Each sample was supplemented with a ×5 loading buffer in a 1:4 ratio and subsequently underwent high temperature denaturation for 10 min. Subsequently, the proteins were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore, MA, United States). Following blocking and washing with TBST, the membranes were exposed to primary and secondary antibodies. Band density quantification was conducted with the aid of Image Lab software (Bio-Rad, CA, United States).

2.13 Statistical analysis

The data from at least three independent experiments were analyzed statistically and expressed as the mean ± standard deviation (SD). GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, United States) was used to analyze the data using

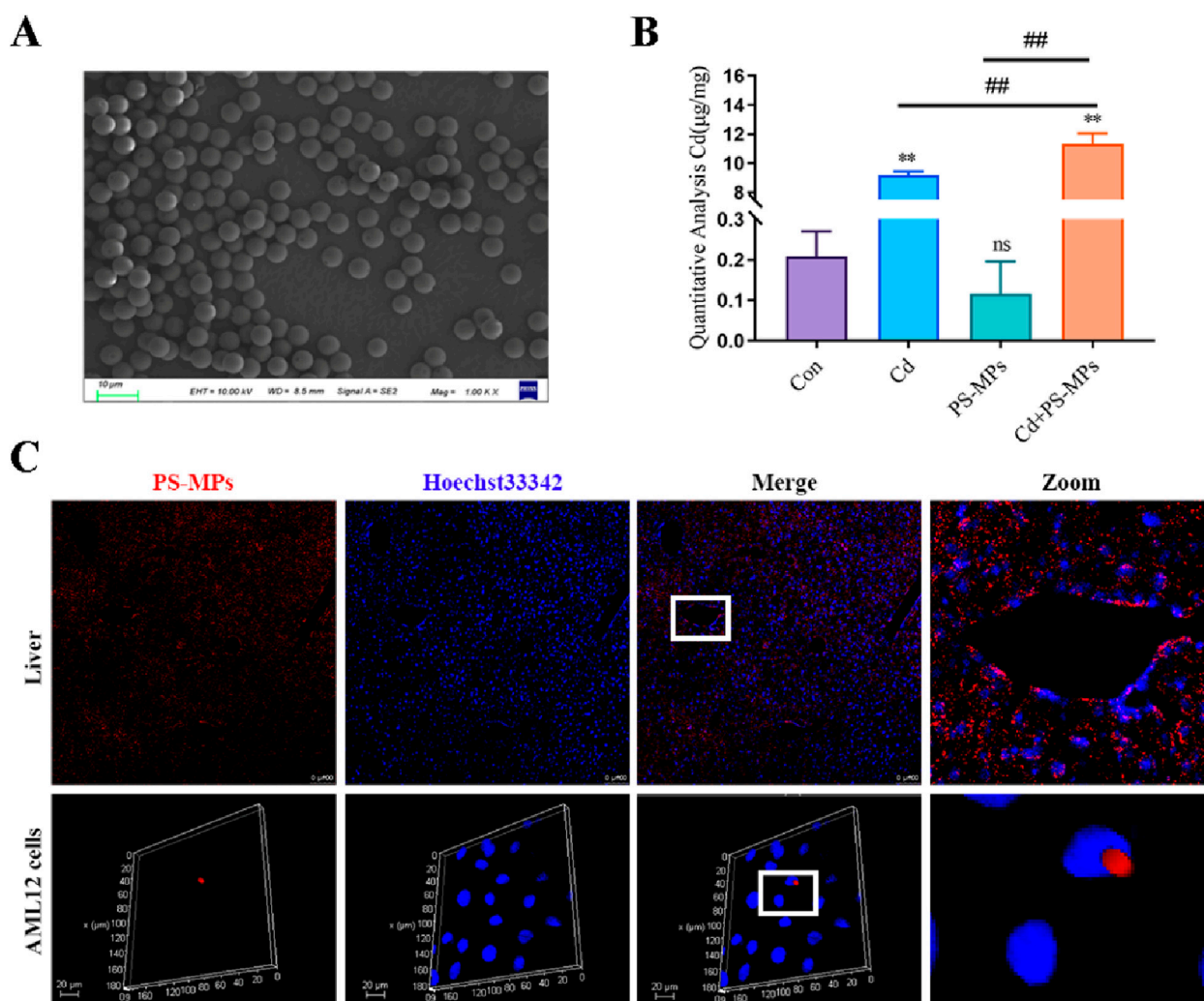


FIGURE 1 PS-MPs accumulate in mouse liver and promote Cd accumulation. Morphology of PS-MPs (A). The concentration of Cd in the liver (B). Levels of PS-MPs in the liver and AML12 (C). Scale bar = 100 μm, Scale bar = 20 μm. Results are shown as the mean ± SD (n = 3). Compared with the control group, * $P < 0.05$, ** $P < 0.01$. Compared with the Cd + PS-MPs group, # $P < 0.05$, ## $P < 0.01$.

one-way analysis of variance [(ANOVA) (Scheffe's SF test)]. P values less than 0.05 indicated a significant difference.

3 Results

3.1 PS-MPs accumulate in mouse liver and promote Cd accumulation

Firstly, we observed the morphology of the PS-MPs using SEM, and the results, as shown in Figure 1A, showed that the PS-MPs were of uniform particle size, with smooth surfaces and regular spherical shapes. In order to further investigate the effect of co-exposure of PS-MPs and Cd on the Cd accumulation profile, Cd levels in the liver were examined in this study, and the results are shown in Figure 1B. Compared to the control group, the Cd content within the Cd group was significantly increased, and when PS-MPs and Cd were co-exposed, it further increased the accumulation of Cd. Subsequently, we also further

examined the accumulation of PS-MPs in the liver, and the results, as shown in Figure 1C, showed that the red fluorescence carried by PS-MPs was observed in both the liver and AML12.

3.2 PS-MPs and Cd co-exposure exacerbate liver injury induced by exposure alone

This study began by measuring changes in body weight and liver coefficient. The results revealed that the Cd and Cd + PS-MPs groups experienced a decrease in body weight compared to the control group. However, there were no changes observed between the Cd + PS-MPs group and the Cd group. Exposure to Cd or PS-MPs did not elicit a significant impact on the liver coefficient; however, their combined exposure resulted in a reduction (Figures 2A, B). Subsequently, TEM was implemented to examine the ultrastructure of hepatocytes. The control group displayed distinct nuclei with regularly distributed chromatin.

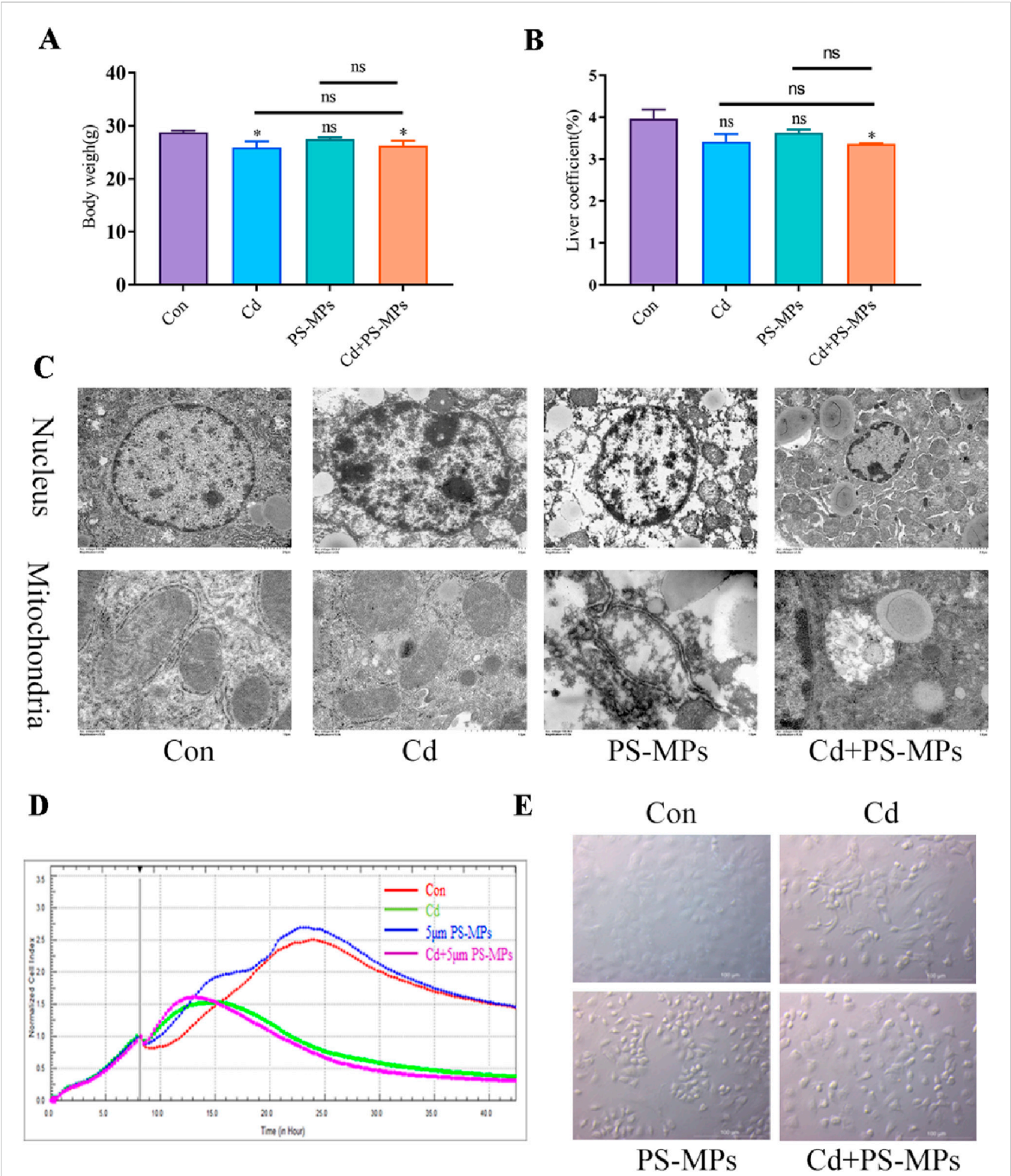


FIGURE 2 PS-MPs and Cd co-exposure exacerbates liver injury induced by exposure alone. Body weights (**A**) and the liver coefficient (**B**) in mice. Transmission Electron Microscope (TEM) observed the ultrastructure of nucleus and mitochondria (**C**). Scale bar = 2 μ m, Scale bar = 0.5 μ m. AML12 cells were observed after exposure of Cd and PS-MPs for 12 h. Cells index was detected by RTCA (**D**). Brightfield observation of cell morphology (**E**). Scale bar = 100 μ m. Results are shown as the mean $\pm$ SD (n = 3). Compared with the control group, * P < 0.05, ** P < 0.01. Compared with the Cd + PS-MPs group, # P < 0.05, ## P < 0.01.

Conversely, the treated group experienced nuclei morphology variation, intensified nuclear chromatin staining, constricted nuclear membrane surrounding the nucleus, and an amplified nuclear pore gap. The Cd + PS-MPs group exhibited more modifications in nuclear morphology, greater nuclear crumpling, and increased intercellular gaps in comparison to the Cd group. On the other hand, the control group showed normal mitochondria structure with well-defined internal and external membranes along with a uniform distribution of cristae. The mitochondria observed in the Cd group appeared to be swollen, deformed, and had a blurred bilayer structure. Furthermore, the mitochondrial cristae in the Cd + PS-MPs group exhibited fractures, dissolution, and vacuolization to a greater extent than those in the Cd group (Figure 2C). We established an *in vitro* model using AML12 cells. The cells were treated with Cd and PS-MPs either independently or in combination. We utilized RTCA technology to obtain real-time measurements of the Cellular index (CI). The findings, as presented in Figure 2D demonstrate that the CI was achieved by the Cd group and Cd + PS-MPs group after 12 h of exposure, followed by a subsequent decline. On the contrary, neither the control group nor the PS-MPs group displayed a decrease in CI until approximately 24 h of exposure. The cells were observed through bright field conditions. The results are presented in Figure 2E, in comparison to the control group, the treated group exhibited indications of wrinkling, wider gaps, and clear alterations in their morphology. Moreover, the cell density was notably reduced in both the Cd group and the PS-MPs group. Overall, co-exposure to PS-MPs and Cd exacerbates liver injury by disrupting hepatocyte morphology and function.

3.3 Co-exposure of Cd and PS-MPs disrupts lipid metabolism and thus exacerbates lipid accumulation

Figure 3A depicts the HE staining results. White, rounded lipid droplets were observed in both the Cd group and the PS-MPs group, either alone or in the combined exposure group. These findings are consistent with observations obtained via TEM (Figure 3B). The kits detected T-CHO, TG, and HDL-C content in the liver tissue of mice. Figures 3C–E presents the results. Compared to the control group, T-CHO content significantly decreased in each treatment group. TG content significantly increased in each treatment group. HDL-C notably increased in the Cd + PS-MPs group and showed a tendency to rise in other groups but with no statistical variance. The lipid metabolism-related proteins were identified by WB. The ACC exhibited a significant increase in the Cd group relative to the control group. Furthermore, in comparison with the Cd group, the Cd + PS-MPs group displayed a significant elevation in ACC levels. The levels of PPAR- α , CPT1, and Apob exhibited a declining trend among all treatment groups as compared to the control group. Moreover, the expression of PPAR- α , CPT1, and Apob was reduced in the Cd + PS-MPs group in contrast to the Cd group (Figure 3F). The experiment also revealed oil red O staining in AML12 cells, confirming lipid droplet accumulation, as illustrated in Figure 4A. A few orange lipid droplets were visible in the control group, while the Cd or PS-MPs groups had

increased amounts of droplets compared to the control group. Additionally, the Cd + PS-MPs group had more lipid droplets. Lipid droplets were detected through fluorescent staining in AML12 cells, as illustrated in Figure 4B. Comparison to the control group, the Cd or PS-MPs group exhibited an increased number of green aggregates along with enhanced fluorescence intensity. The Cd + PS-MPs group demonstrated a further increase in the number of green aggregates and their fluorescence intensity in comparison to the Cd or PS-MPs groups. The kits detected T-CHO and TG content in the AML12 cells. Figures 4C, D displays the results. Compared to the control group, all treatment groups had significantly higher levels of T-CHO and TG. The Cd + PS-MPs group demonstrated a significant increase in T-CHO and TG content compared to the Cd group. Changes in the expression of ACC, CPT1, and PPAR- α showed consistency with the *in vivo* experiments (Figure 4E). The co-exposure of Cd and PS-MPs has been identified as a key factor in the promotion of lipid accumulation, with the inhibition of CPT1 expression emerging as a key mechanism through which this occurs.

3.4 Combined exposure to Cd and PS-MPs exacerbates exposure alone-induced blockage of autophagic flux

TEM examination revealed the presence of autophagosomes in mouse liver as illustrated in Figure 5A. In comparison to the control group, autophagosomes featuring a double-membrane structure coalesced with lipid droplets were noted in the treatment group. There were higher incidences of autophagosomes in the Cd + PS-MPs group than in those treated with Cd or PS-MPs. In comparison to the control group, there was a tendency for an increment in the expression of P62, LC3, ATG7, and Beclin1 in all treatment groups. When compared to the Cd group, the expression of P62, LC3, ATG7, and Beclin1 increased significantly in the Cd + PS-MPs group (Figure 5B). Autophagic flux detection using RFP-GFP-LC3 in AML12. The results indicate an increase in yellow fluorescent aggregates in the Cd group along with their brightness, while denser yellow aggregates emerged in the PS-MPs group compared to the control group. More and brighter yellow fluorescent aggregates were observed in the Cd + PS-MPs group in comparison to the Cd group (Figure 5C). The results are showcased in Figure 5D. The expression of P62, LC3, and ATG7 was extremely increased in all treatment groups when compared to the control group. Compared to the Cd group, the expression of LC3 and ATG7 was higher in the Cd + PS-MPs group. Additionally, the expression of P62 showed a tendency to increase, although this was not statistically significant.

3.5 Activation of CPT1 alleviates PS-MPs and Cd co-exposure-induced blockage of autophagic flux

The results are presented in Figure 6A. It was observed that the control and Baicalin groups had minimal yellow aggregates, whereas the yellow aggregates in the Cd + PS-MPs group increased in

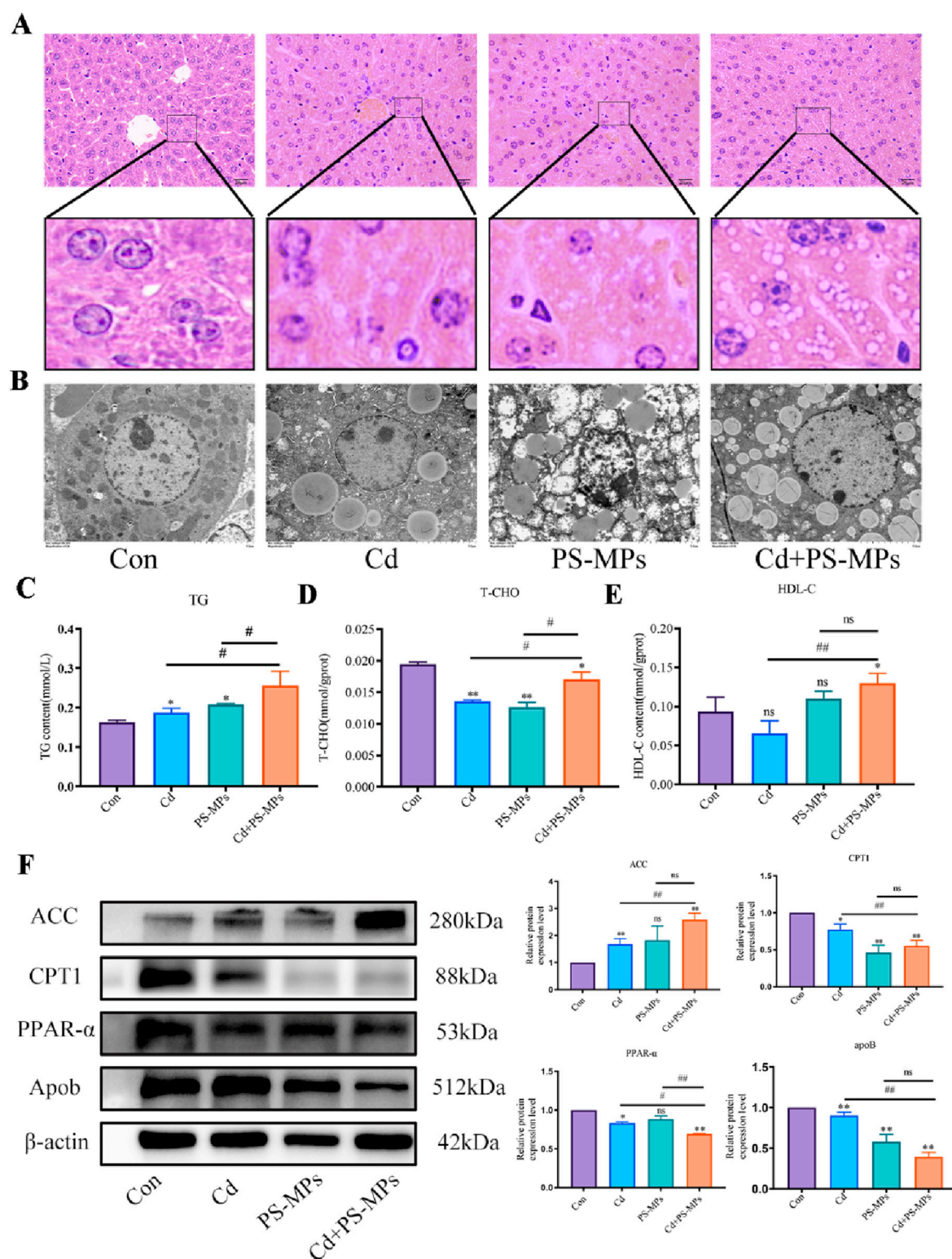
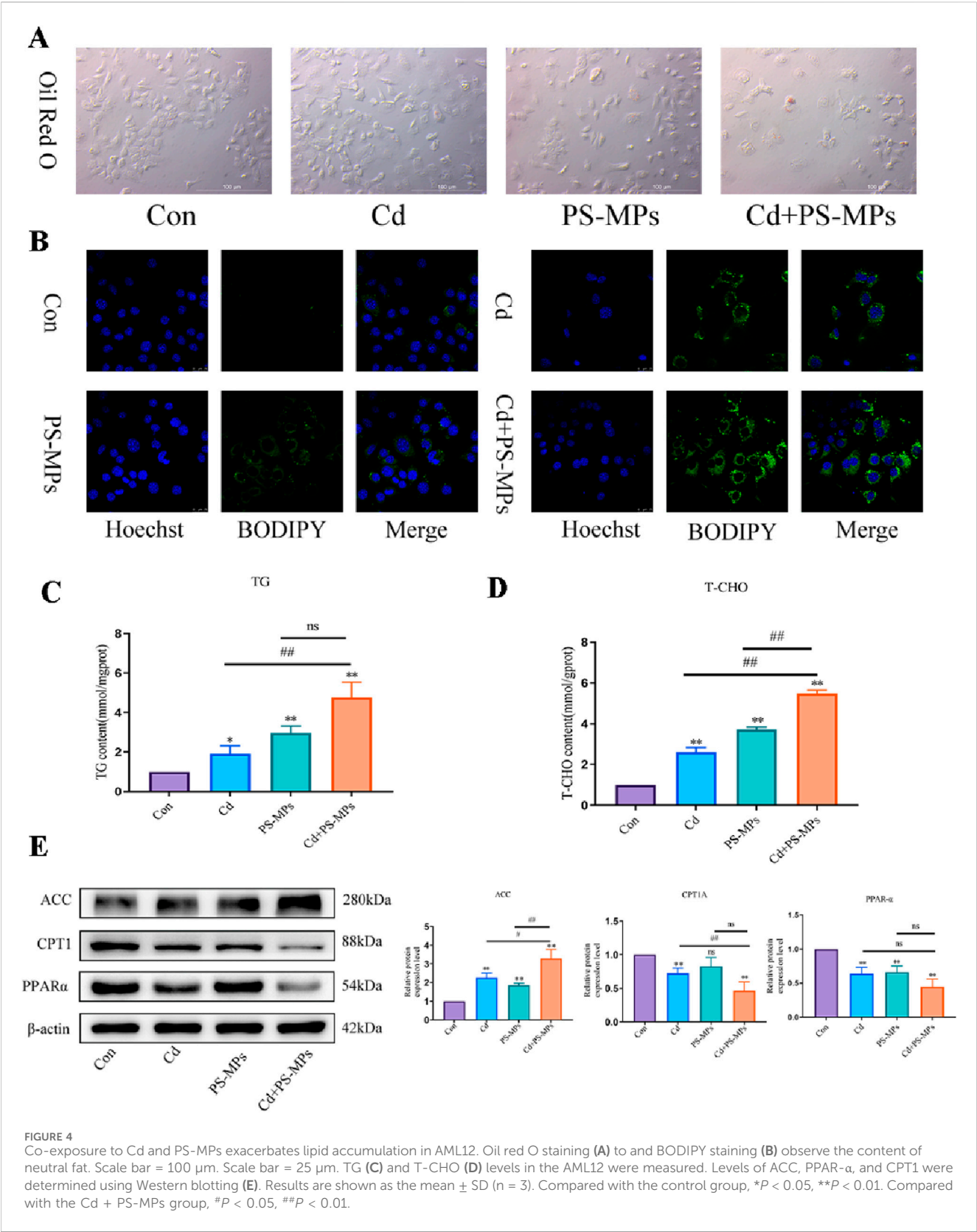


FIGURE 3

Co-exposure to Cd and PS-MPs exacerbates lipid accumulation in mice. Liver histopathology observed using HE staining (A). Scale bar = 20 μ m. TEM observed the ultrastructure of lipid droplets (B). Scale bar = 5 μ m. TG (C), T-CHO (D), and HDL-C (E) levels in the mice were measured. Levels of ACC, CPT1, PPAR- α , and Apob were determined using Western blotting (F). Results are shown as the mean $\pm$ SD (n = 3). Compared with the control group, * P < 0.05, ** P < 0.01. Compared with the Cd + PS-MPs group, # P < 0.05, ## P < 0.01.



number and brightness. The yellow aggregates in the Baicalin + Cd + PS-MPs group were significantly reduced. In comparison to the control group, the expression of ATG7, P62, and LC3 was higher in the Cd + PS-MPs group. Conversely, in the Baicalin + Cd + PS-MPs group, the expression of ATG7, P62, and LC3 was significantly lower in comparison with the Cd + PS-MPs group (Figure 6B).

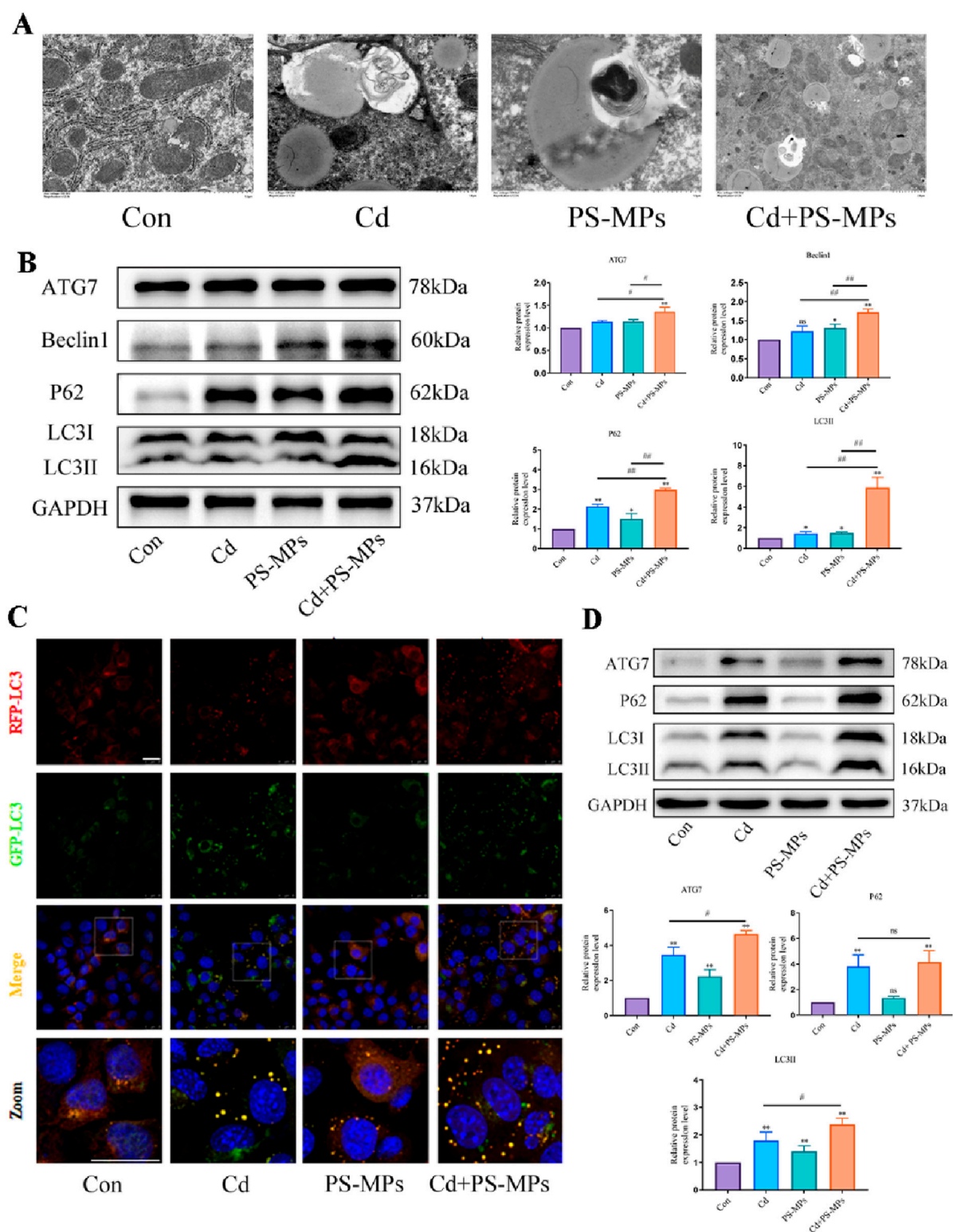
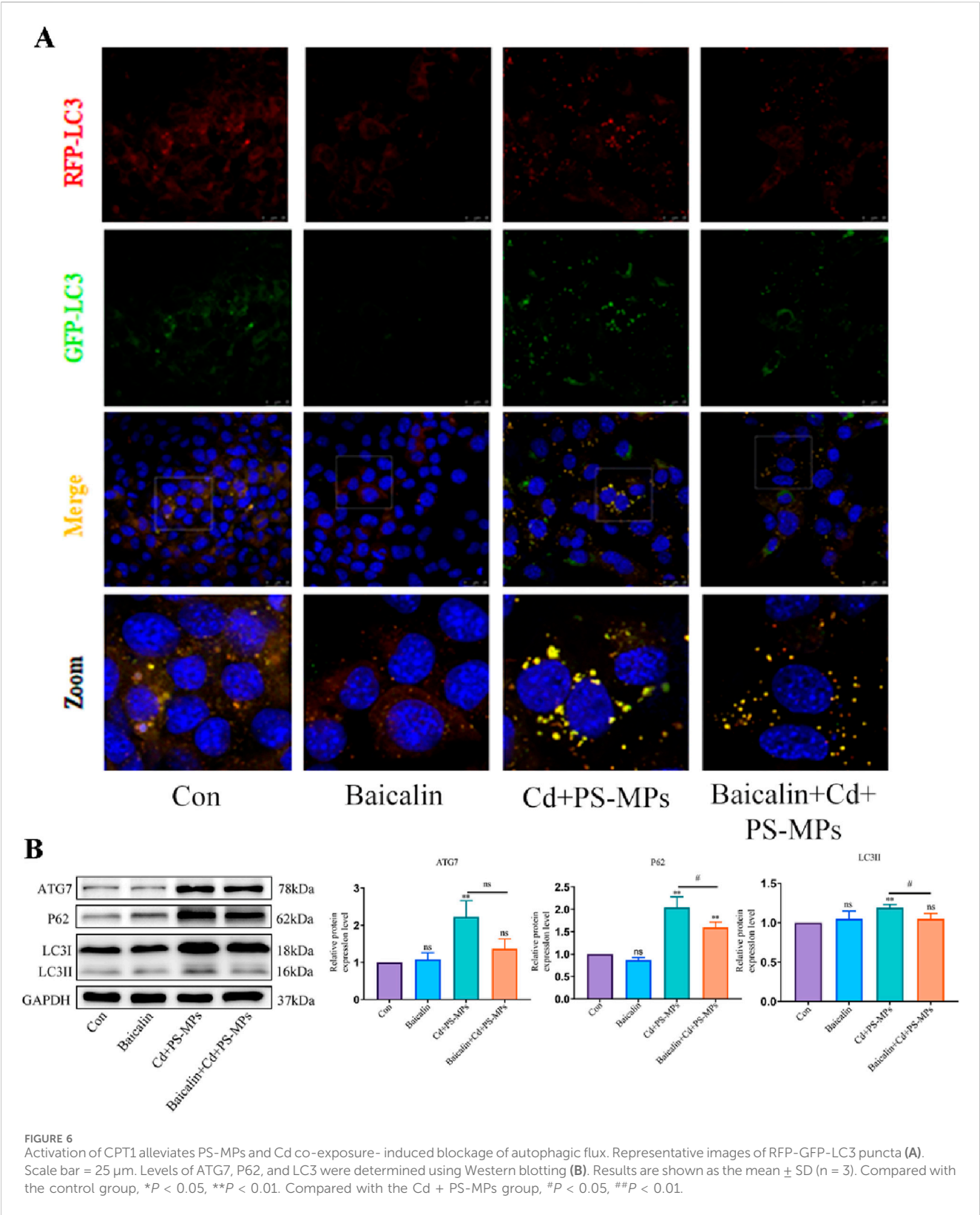


FIGURE 5

Co-exposure to Cd and PS-MPs exacerbates exposure alone-induced blockage of autophagic flux. TEM observation of hepatocyte autophagosomes (A). Scale bar = 2 μ m. Levels of ATG7, Beclin1, P62, and LC3 were determined using Western blotting (B). AML12 cells were observed after exposure of Cd and PS-MPs for 12 h. Representative images of RFP-GFP-LC3 puncta (C). Scale bar = 25 μ m. Levels of ATG7, P62, and LC3 were determined using Western blotting (D). Results are shown as the mean $\pm$ SD (n = 3). Compared with the control group, * P < 0.05, ** P < 0.01. Compared with the Cd + PS-MPs group, # P < 0.05, ## P < 0.01.



3.6 Relief of autophagic flux blockade reduces liver lipid accumulation

In the Baicalin + Cd + PS-MPs groups, 100 μ mol/L baicalin was added beforehand followed by exposure to Cd and PS-MPs for 12 h

after 1 h. The outcomes were exhibited in Figure 7A. The results indicate that the number of lipid droplets was minimal in both the control and Baicalin groups, in comparison to a significant increase in lipid droplets observed in the Cd + PS-MPs group. The Baicalin + Cd + PS-MPs group exhibited a considerable decrease in lipid droplet content

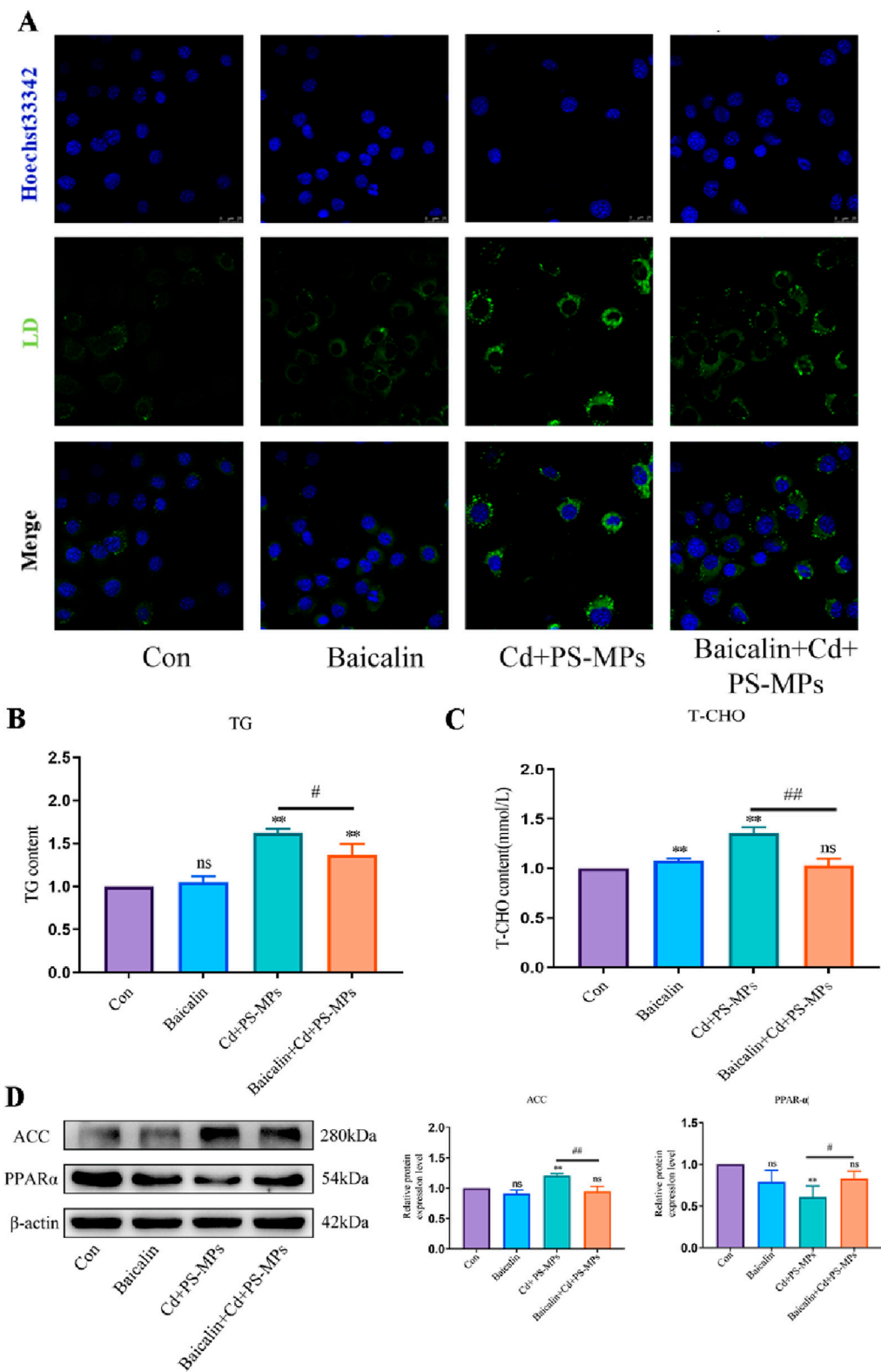


FIGURE 7 Activation of CPT1 alleviates PS-MPs and Cd co-exposure-induced lipid accumulation. BODIPY staining observe the content of neutral fat (A). Scale bar = 25 μ m. TG (B) and T-CHO (C) levels in the AML12 were measured. Levels of ACC and PPAR- α were determined using Western blotting (D). Results are shown as the mean $\pm$ SD (n = 3). Compared with the control group, * P < 0.05, ** P < 0.01. Compared with the Cd + PS-MPs group, # P < 0.05, ## P < 0.01.

relative to the Cd + PS-MPs group. The content of T-CHO and TG was identified, with the outcomes presented in Figures 7B, C. The levels of T-CHO and TG were increased in the Cd + PS-MPs group compared to the control group. Conversely, the Baicalin + Cd + PS-MPs group displayed a significant decrease in T-CHO and TG levels relative to the Cd + PS-MPs group. When compared to the control group, the Cd + PS-MPs group displayed a notable rise in ACC and a decrease in PPAR- α expression. Additionally, when compared to the Cd + PS-MPs group, the Baicalin + Cd + PS-MPs group exhibited an increase in PPAR- α expression and a decrease in ACC expression (Figure 7D). Overall, restoration of autophagic flux alleviated lipid accumulation induced by co-exposure to PS-MPs and Cd.

4 Discussion

Cd pollution is a significant public health concern on a global scale (Wang et al., 2015). Cd can be found in a range of sources, such as water, soil, food, and tobacco products, and can enter the body via multiple pathways. The increasing worldwide production of MPs has generated significant apprehension over their potential contamination, and their impact on animal health has drawn attention across the world (Chen et al., 2021; Horton et al., 2017). MPs are ubiquitous in the air, soil, water, and many food and household items. They penetrate the organism through the digestive, respiratory, and dermal routes, causing harm (Yin et al., 2022). PS-MPs is a highly prolific plastic and one of the prevailing MPs types observed in the environment (Lu et al., 2016). The liver is a crucial detoxifying organ in mammals and is sensitive to several toxic agents (Meżyńska et al., 2018; Saïdi et al., 2013). Studies have demonstrated that PS-MPs and Cd frequently target the liver (Meżyńska and Brzóska, 2019; Goodman et al., 2022). The current study demonstrated that PS-MPs intensified the accumulation of Cd in the liver of mice, aligning with the findings of Lu et al. (2018a)'s zebrafish tissue experiment on the impact of PS-MPs on Cd accumulation. Fluorescent PS-MPs were orally administered to mice, and subsequent liver sections were examined. Results revealed that PS-MPs accumulated in the liver with a more uniform distribution. Likewise, exposure of AML12 cells to PS-MPs for 12 h displayed the presence of PS-MPs in the cells, mainly concentrated around the nucleus. These findings demonstrate the accumulation of PS-MPs in the liver cells. The findings indicate that PS-MPs and Cd are capable of entering hepatocytes, providing a basis for further investigation into the toxicity mechanism of PS-MPs and Cd. The study also analyzed the influence of Cd and PS-MPs on mouse weight and liver index revealing significant reductions post Cd exposure, following Brzóska's report (Brzóska et al., 2003). The body weight of mice was significantly reduced following exposure to PS-MPs, which is in line with Lu et al. (2018b) and Jin et al. (2021)'s findings. Although exposure to PS-MPs alone had no statistically significant effect on the liver coefficient of mice, it is noteworthy that the Cd + PS-MPs group had the lowest liver coefficient. This suggests that the joint action of Cd and PS-MPs on the liver resulted in a greater toxicity of Cd, while simultaneously worsening the liver damage through the intensification of the PS-MPs. This led to necrosis and deformation of certain liver regions, ultimately culminating in a reduction of the liver coefficient. The HE staining observations of pathological sections of the liver and TEM observations of the ultrastructure of liver cells confirmed the damage inflicted by exposure to Cd and PS-MPs. The bodily harm includes liver cord

ailments, nucleus crumpling, mitochondrial cristae rupture, and vacuolisation.

The liver regulates lipid homeostasis by absorbing fatty acids, undergoing *de novo* lipogenesis, producing triglycerides, and dividing fatty acids (Jones, 2016). The influence of Cd exposure on liver lipid metabolism remains insufficiently researched. In their examination of Cd-exposed zebrafish, Pan et al. (2018) noted that Cd-induced oxidative stress and mitochondrial malfunction resulted in liver lipid accumulation. Wu et al. (2017) found that Cd concentrations at 200 $\mu\text{g/L}$ and 500 $\mu\text{g/L}$ can impact the lipid metabolism of toad liver. Furthermore, Cd can also disrupt the liver's lipid metabolic homeostasis in animal models involving mice and rats, leading to lipid accumulation in the liver (Zhang et al., 2018; Go et al., 2015). Research into the effects of PS-MPs exposure on liver lipid metabolism is currently limited. Lu et al. (2016) observed the accumulation of PS-MPs in the livers of zebrafish after 7 days of exposure, leading to disruption of liver lipid and energy metabolism. Similar findings were made by Lu et al. (2018b) and Deng et al. (2017). Cheng and colleagues researched the harmful effects of PS-MPs on the human liver with the aid of LOs, which are liver-like organs produced by stem cells, as a substitute model. Their conclusions revealed that PS-MPs impeded the expression of genes and protein synthesis of PPAR α , while increasing lipid accumulation in LOs (Cheng et al., 2022). In this experimental study, we found that Cd and PS-MPs exposure significantly increased the content of TG in liver tissues, and PS-MPs exacerbated the Cd-induced TG elevation. The above results suggest that Cd and PS-MPs exposure caused abnormal lipoprotein metabolism, which led to lipid accumulation in the liver, and PS-MPs exacerbated the lipotoxicity of Cd on the liver to a certain extent. The results of HE staining and TEM observation of the ultrastructure also proved that. ACC and FAS are key proteins for lipid synthesis. β -oxidation is the main pathway for fatty acid consumption by liver, and the main process was regulated by PPAR α regulation, and CPT1 assists fatty acyl coenzyme A to enter the mitochondria and is a key enzyme in β -oxidation (Alves-Bezerra and Cohen, 2017). The results of the present study showed that exposure to Cd and PS-MPs elevated the expression of ACC and decreased the expression of PPAR α , CPT1, and Apob, and the presence of PS-MPs exacerbated the Cd-induced abnormalities of lipid metabolism, which was in accordance with the previous results. Elevated expression of ACC further inhibited the expression of CPT1 and suppressed the rate of β -oxidation, which resulted in the ability of lipid degradation within the liver to be Reduced. Oil red O staining, lipid droplet fluorescence staining, T-CHO, TG and lipid metabolism-related proteins were performed on AML12 cells, and the results were consistent with *vivo* studies.

Autophagy is a cellular regulatory mechanism, present naturally, which preserves the stability of the intracellular environment by breaking down toxic components and damaged organelles (Niture et al., 2021). Several experimental studies on the Cd-induced autophagy in liver have been performed, demonstrating the enhancement of autophagy levels and hindrance of autophagic flux in liver (Duan et al., 2023) (Sun et al., 2022). In a survey conducted on freshwater fish, it was discovered that PS-MPs elicit autophagy and apoptosis via disruption of cellular signaling. Zhong et al. (2022) revealed that the co-exposure to arsenic and nanoplastics induces excessive autophagy by modifying the levels of PI3K, mTOR, Beclin1, ATG5, LC3, and P62 expression. In this study, TEM

analysis revealed the presence of autophagic vesicles in the liver tissues of groups exposed to Cd and PS-MPs. These vesicles were found near or involved in the degradation of lipid droplets, consistent with previous findings by Singh et al. (2009) regarding the involvement of autophagy pathways in lipid droplet metabolism. There is a significant rise in the expression of ATG7, P62, Beclin1, and LC3 in the groups exposed to Cd and PS-MPs, as suggested by the findings. Later, the effects of Cd and PS-MPs on autophagic flux were detected. Yellow fluorescent aggregates were observed in Cd and PS-MPs groups. The yellow aggregates of the Cd + PS-MPs group were brighter, indicating a more severe blockage of autophagic flux. In summary, the exposure to cadmium and PS-microplastics resulted in the blockage of autophagic flux in mouse liver and AML12 cells. Furthermore, PS-MPs exacerbated the Cd-induced blockage of autophagic flux.

The main pathway for fat depletion is through fatty acid β -oxidation in mitochondria, which is divided into three main stages: activation of fatty acids, transfer of lipoyl coenzyme A and β -oxidation of lipoyl coenzyme A (Houten et al., 2016). The long-chain lipoyl coenzyme A produced by esterification in the cytosol in the first stage has to enter the mitochondrial matrix for oxidation, at which point CPT1 is required to mediate it (Nees et al., 2015). CPT1 is therefore the key rate-limiting enzyme for fatty acid β -oxidation (Bonnefont et al., 2004). It has been shown that increasing CPT1 activity effectively enhances the rate of β -oxidation and improves lipid accumulation in the liver (Dai et al., 2018). Lipid metabolism also affects autophagy. Mikami et al. (2012) induced mice with a high-fat diet and observed an increase in autophagic vesicles and a decrease in autophagic flux in WAT of obese mice. Similar conclusions were reached that an abnormal increase in lipids leads to blockage of autophagic flux (Xie et al., 2020). This interrelationship can trap hepatocytes in a deleterious cycle whereby lipid accumulation affects autophagy and autophagy inhibition further affects lipid degradation, leading to lipid accumulation in the liver. Therefore, we postulated whether CPT1 could impact autophagy and ultimately reduce lipid accumulation. Baicalin was utilized to activate CPT1, while AML12 cells underwent exposure to Cd and PS-MPs for 12 h in this investigation. Findings showed that the addition of baicalin significantly reduced lipid accumulation in the group. Activation of CPT1 by baicalin resulted in a marked reduction in the expression of ACC as well as a significant increase in the expression of PPAR α , indicating a potential acceleration of β -oxidation. The expression of P62 and LC3 significantly decreased. The RFP-GFP-LC3 tandem fluorescent protein assay results presented a significant reduction of yellow fluorescent spots after the addition of baicalin. This suggests that there was smooth fusion between autophagosomes and lysosomes, and fluent autophagic flux.

5 Conclusion

Overall, both Cd and PS-MPs enter and accumulate in mouse and AML12 cells. Exposure to Cd and PS-MPs, alone or in combination, resulted in significant decreases in body weight and liver coefficients in mice. Exposure to PS-MPs exacerbated Cd liver damage to some extent. Exposure to Cd or PS-MPs increased fatty acid production and impeded lipid metabolism, leading to atypical lipid accumulation. PS-MPs exacerbated Cd-induced lipid accumulation. In combination with Cd and PS-MPs, CPT1 is a

key target for inducing lipid accumulation. Activation of CPT1 significantly increased the β -oxidation of fatty acids and alleviated the blockage of autophagic flux, thereby reducing the hepatotoxicity induced by the combined exposure to Cd and PS-MPs. This study demonstrated the mechanism of hepatotoxicity induced by Cd and PS-MPs, and provided a new theoretical basis for the prevention and treatment of liver injury and lipid accumulation.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by the Yangzhou University Experimental Animal Welfare Ethics Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ZC: Conceptualization, Investigation, Writing—original draft. HQ: Conceptualization, Investigation, Writing—original draft. JS: Conceptualization, Investigation, Writing—original draft. TW: Data curation, Methodology, Writing—review and editing. YY: Data curation, Visualization, Writing—review and editing. JG: Data curation, Methodology, Writing—review and editing. JB: Writing—review and editing. ZL: Writing—review and editing. HZ: Conceptualization, Investigation, Software, Writing—original draft.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was supported by the National Key R&D Program of China (No. 2023YFD1801100), the National Natural Science Foundation of China (Nos 32072933, and 32273086), 111 Project D18007, a Project Funded by Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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OPEN ACCESS

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RECEIVED 14 November 2024

ACCEPTED 23 December 2024

PUBLISHED 09 January 2025

CITATION

Liu Z, Gan Y, Shen Z, Cai S, Wang X, Li Y, Li X,
Fu H, Chen J and Li N (2025) Role of copper
homeostasis and cuproptosis in heart failure
pathogenesis: implications for
therapeutic strategies.
Front. Pharmacol. 15:1527901.
doi: 10.3389/fphar.2024.1527901

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Role of copper homeostasis and cuproptosis in heart failure pathogenesis: implications for therapeutic strategies

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Copper is an essential micronutrient involved in various physiological processes in various cell types. Consequently, dysregulation of copper homeostasis—either excessive or deficient—can lead to pathological changes, such as heart failure (HF). Recently, a new type of copper-dependent cell death known as cuproptosis has drawn increasing attention to the impact of copper dyshomeostasis on HF. Notably, copper dyshomeostasis was associated with the occurrence of HF. Hence, this review aimed to investigate the biological processes involved in copper uptake, transport, excretion, and storage at both the cellular and systemic levels in terms of cuproptosis and HF, along with the underlying mechanisms of action. Additionally, the role of cuproptosis and its related mitochondrial dysfunction in HF pathogenesis was analyzed. Finally, we reviewed the therapeutic potential of current drugs that target copper metabolism for treating HF. Overall, the conclusions of this review revealed the therapeutic potential of copper-based therapies that target cuproptosis for the development of strategies for the treatment of HF.

KEYWORDS

copper, copper homeostasis, cuproptosis, mitochondrion, heart failure

1 Introduction

Heart failure (HF) is one of the most prevalent cardiovascular diseases worldwide and poses a significant threat to human health (Zannad, 2018). It is characterized by impaired cardiac function due to ventricular filling and ejection dysfunction and represents the end stage of various cardiovascular disorders (Ziaeian and Fonarow, 2016). Despite ongoing advancements in treatment methods, the prognosis for patients with HF remains poor, making it a serious public health concern (Ambrosy et al., 2014). Furthermore, HF places a substantial economic burden on healthcare systems (Ziaeian and Fonarow, 2016). Consequently, a thorough understanding of the potential therapeutic targets and underlying mechanisms of HF is crucial for improving its prognosis and patient outcomes.

Copper is an essential micronutrient and a vital catalytic cofactor involved in various biological processes, including the production of biomolecules, antioxidant defense, and mitochondrial respiration (Chen L. et al., 2021; Maung et al., 2021). Copper homeostasis is tightly regulated, as both excessive and deficient levels of copper can lead to pathological changes that adversely affect human health (Maung et al., 2021). Dysregulation of copper homeostasis can contribute to the pathogenic mechanisms of HF by influencing inflammation (Wang et al., 2022), oxidative stress (Tsutsui et al., 2011; Vo et al., 2020), energy metabolism (Fan et al., 2005), cell death (Vanempel et al., 2005; Jiang et al., 2022), responses to β -adrenergic stimulation (Elsherif et al., 2004b), and calcium homeostasis (Elsherif et al., 2007). Additionally, a newly identified pattern of copper-dependent cell death, namely, cuproptosis, may facilitate the occurrence of HF by affecting mitochondrial function.

This review aimed to investigate the roles of copper homeostasis and cuproptosis in HF, highlighting their potential for the development of therapeutic strategies for HF by targeting cuproptosis. The conclusions of this review may provide insights into future research directions regarding the relationship between cuproptosis and HF.

2 Copper homeostasis biochemical and molecular insights

2.1 Systemic copper metabolism

In the field of “metals in biology,” metals play unique and crucial roles in biological systems. Copper is an essential trace metal found in nearly every living organism, with a normal human body containing approximately about 100 mg (Wang D. et al., 2023). It is primarily found in muscles, bones, and the liver, with small quantities present in the blood (Festa and Thiele, 2011). Copper exists in two distinct ionic forms, namely, cuprous ions (Cu [I], reduced type) and cupric ions (Cu [II], oxidized type), both of which are involved in the enzymatic modulation of cellular physiological activities. However, redox cycling between Cu(I) and Cu(II) can contribute to the catalytic generation of highly toxic hydroxyl radicals, subsequently damaging macromolecules (Halliwell and Gutteridge, 1984). Therefore, it is necessary to maintain systemic copper levels within a stable range to ensure proper biochemical processes and prevent cellular damage (Figure 1).

2.1.1 Copper uptake

Copper is acquired primarily from dietary sources such as meat, offal, nuts, and cereals (Bost et al., 2016). The absorption of dietary copper, predominantly in the form of Cu(II), occurs mainly in the small intestine (Mason, 1979) by intestinal epithelial cells and is modulated by copper transporter 1 (CTR1), which is encoded by the solute carrier family 31 member 1 (SLC31A1) gene and located on the apical surface of the cells (Petris, 2004). Reportedly, CTR1 is crucial for absorption, and its systemic or tissue-specific deletion significantly reduces dietary copper absorption (Lee et al., 2002). Additionally, the activities of duodenal cytochrome b (DCYTB) and six-transmembrane epithelial antigen of the prostate (STEAP) facilitate this process by reducing Cu(II) to Cu(I) (Dancis et al.,

1992; Georgatsou et al., 1997), the ionic state that is transported by CTR1.

2.1.2 Copper transport and storage

Following copper absorption through intestinal epithelial cells, it is secreted into the bloodstream and binds soluble chaperones such as ceruloplasmin (CP), human serum albumin, macroglobulin, and histidine (Moriya et al., 2008; Ramos et al., 2016; Lutsenko, 2021). These complexes transport copper to the liver via the portal vein, where hepatocytes take up copper through CTR1. Within hepatocytes, copper can either be transported to specific proteins via copper chaperones or chelated by the copper-binding protein metallothionein (MT) for storage (Luza and Speisky, 1996; Krężel and Maret, 2017). Thus, the liver serves as the primary organ responsible for capturing, distributing, and excreting copper, playing a crucial role in regulating systemic copper homeostasis. The ATPase copper transporter 7A (ATP7A) and ATPase copper transporter 7B (ATP7B) mediate copper transport in peripheral and liver tissues, respectively (Wang et al., 2011; Telianidis et al., 2023). ATP7A facilitates the transport of copper to the portal vein, whereas ATP7B is responsible for pumping copper back into the bloodstream from the liver. In the blood, copper ions can again bind soluble chaperones, allowing them to be delivered to various organs and tissues where they catalyze reactions involved in numerous physiological processes (Lutsenko et al., 2007b; La Fontaine et al., 2010).

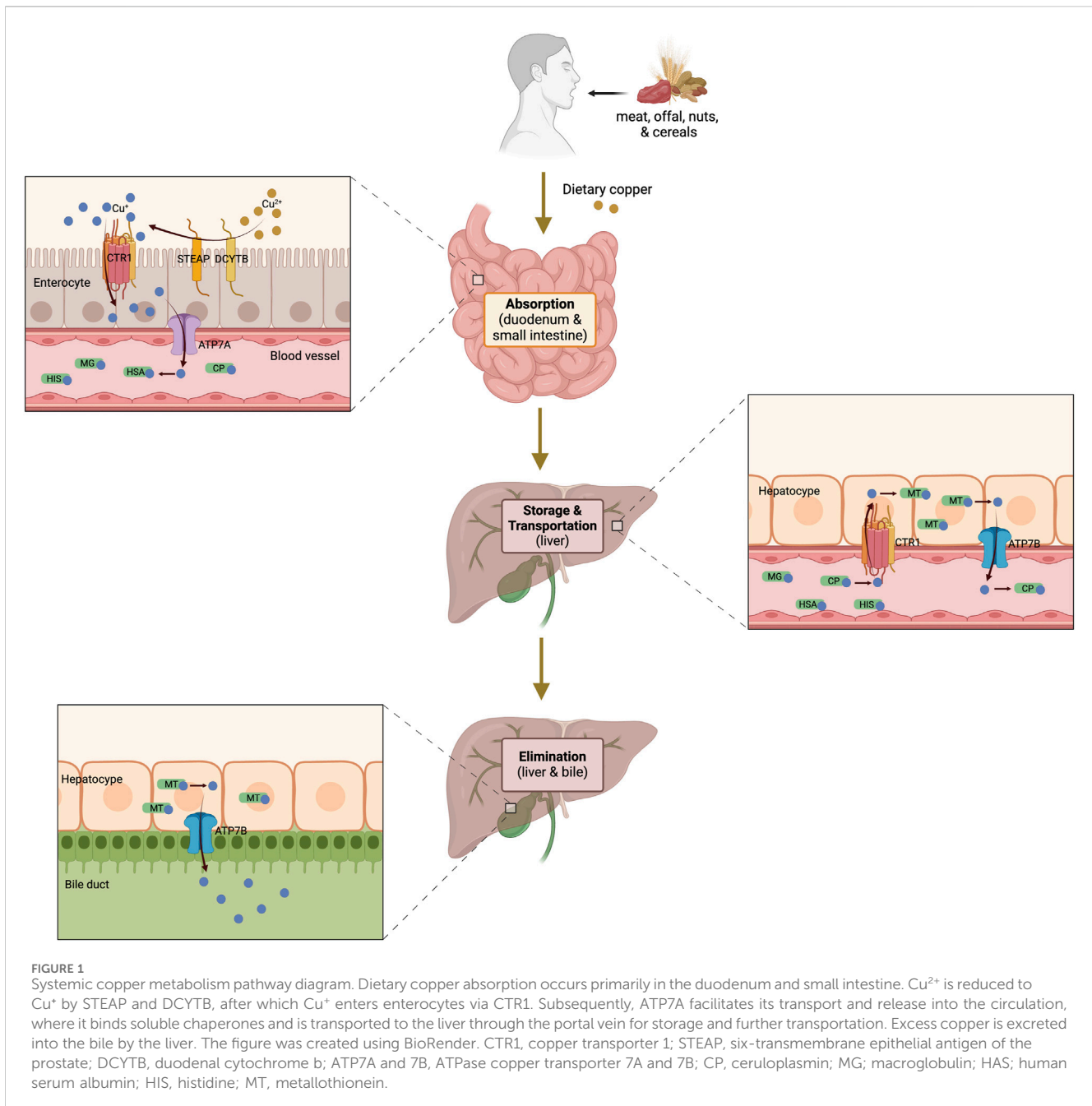
2.1.3 Copper elimination

Excess endogenous copper is primarily excreted through bile and subsequently eliminated in feces (Turnlund, 1998). Other routes, such as sweat, urine, or menstruation, have a lesser impact on copper excretion or depletion. ATP7B plays a crucial role in removing excess copper from the body, and its inactivation (such as in Wilson's disease) can lead to copper accumulation in the liver and subsequent copper-induced toxicity (Yang et al., 2023). Notably, the excretion of endogenous copper has been reported to be significantly affected by dietary copper intake (Turnlund et al., 1989; Scott and Turnlund, 1994).

Taken together, the processes of copper absorption, storage, transport, and elimination in organisms determine the distribution and modulation of copper homeostasis in the body.

2.2 Copper homeostasis is tightly regulated within cells

Copper is essential for cellular activity, and its intracellular concentration is meticulously regulated to prevent the detrimental effects of both copper deficiency and overload (Dupont et al., 2011; Kim et al., 2013). Copper homeostasis and compartmentalization are mediated by a finely tuned network of copper transport proteins, soluble chaperones, copper enzymes, and copper-dependent transcriptional regulators (Burkhead et al., 2009; Robinson and Winge, 2010; Festa and Thiele, 2011). The levels of copper are maintained within a narrow range through the synergistic actions of these copper-dependent proteins (Figure 2).

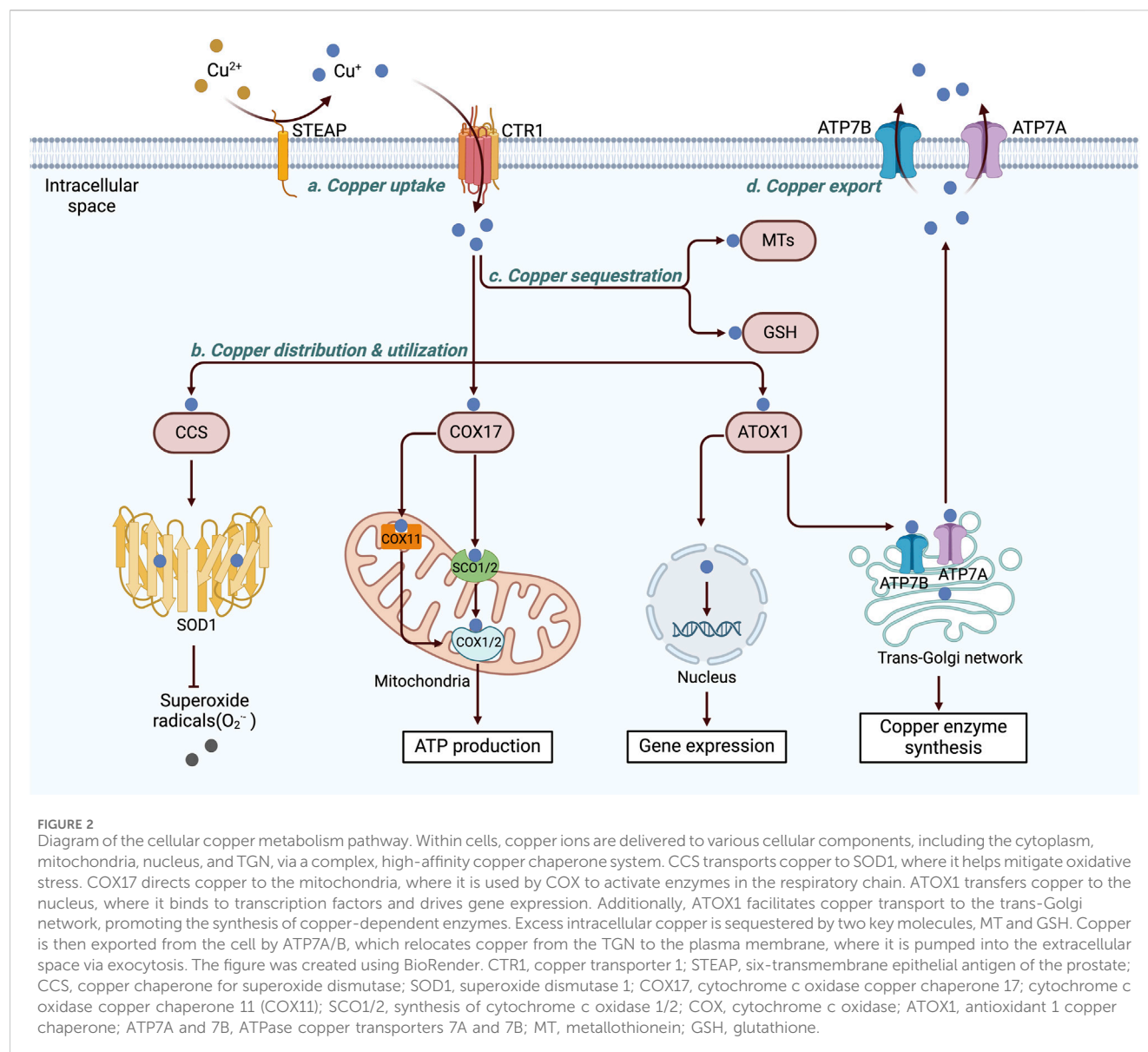


2.2.1 Copper absorption

The high-affinity copper transporter CTR1 (encoded by *SLC31A1*) is a transmembrane protein that forms a stable trimeric channel (Ren et al., 2001), facilitating the absorption of most copper ions in cells by allowing their passage across the plasma membrane (Maryon et al., 2013; Bian et al., 2023). *In vitro* studies have shown that the expression of CTR1 can be modulated in a copper-dependent manner and that it is upregulated under copper-depleted conditions to increase copper uptake and downregulated under copper-overloaded conditions to prevent copper cytotoxicity (Liang et al., 2012; Maryon et al., 2013). CTR1 plays a critical role in maintaining copper homeostasis.

2.2.2 Intracellular copper distribution and utilization

Copper is utilized in various cellular compartments, and its intracellular distribution varies according to metabolic requirements (Lutsenko, 2021). Once copper enters the cell, it is allocated by copper chaperones to specific protein targets within intracellular structures such as the trans-Golgi network (TGN), mitochondria, or nucleus. Three main copper chaperones have been identified within mammalian cells: antioxidant 1 copper chaperone (ATOX1), cytochrome c oxidase (COX) copper chaperone 17 (COX17), and copper chaperone for superoxide dismutase (CCS) (Finney and O'Halloran, 2003; Banci et al., 2010).



ATOX1 can deliver copper to ATP7A and ATP7B within the TGN, facilitating the synthesis of copper-dependent enzymes such as lysyl oxidase (LOX), CP, and tyrosinase (Petrís et al., 2000; Hellman et al., 2002; Shanbhag et al., 2019). Additionally, ATOX1 functions as a copper-dependent transcription factor, transporting copper into the nucleus and promoting cell proliferation (Itoh et al., 2008). It collaborates with specificity protein 1 and metal-regulatory transcription factor 1 to regulate copper-dependent gene expression (Selvaraj et al., 2005; Liang et al., 2012).

CCS transfers copper to superoxide dismutase 1 (SOD1), aiding in the detoxification of reactive oxygen species (ROS) while maintaining copper homeostasis (Rae et al., 2024). The metabolism of oxygen in mitochondria is associated with the production of superoxide, which damages cells. SOD1 functions as a key antioxidant enzyme, converting superoxide radicals into molecular oxygen and hydrogen peroxide (Leitch et al., 2009). CCS and SOD1 are colocalized and interact within various cell types (Casareno et al., 2024; Rae et al., 2024; Rothstein et al., 2024);

however, the exact mechanism of their concurrent transportation to the mitochondria remains unclear (Suzuki et al., 2000).

COX17 transports copper from the cytosol to the mitochondrial inner membrane, facilitating the synthesis of COX (SCO1 and SCO2) and promoting the insertion of copper into the mitochondrially encoded COX subunit 2 (COX2) (Stiburek et al., 2024). Additionally, copper can be transported via COX17 to COX1 from the cytoplasm through COX11 (COX11) (Hiser et al., 2000). Both COX1 and COX2 contain redox-active copper centers that play crucial roles in electron transfer within complex IV, ultimately promoting ATP generation (Nýřltová et al., 2000). Mutations in SCO1, SCO2, and COX17 are associated with reduced COX activity, leading to mitochondrial dysfunction and oxidative stress (Takahashi et al., 2002; Leary et al., 2004).

2.2.3 Intracellular sequestration

Within cells, labile copper can produce ROS and cause cytotoxicity. This harmful process is effectively mitigated by

TABLE 1 Evidence linking copper excess and HF.

Country, author, year	Methods and study population	Result
Greece, Alexanian et al. (2014)	125 patients with AHF or CHF (71% male, aged 69 ± 11 years) and 21 healthy volunteers matched controls	Serum Copper was increased both in AHF ($p = 0.006$) and CHF ($p = 0.002$) and correlated with left ventricular systolic and diastolic function
India, Singh et al. (1985)	44 cases of AMI and 23 cases of angina and 40 age and sex matched healthy controls; HF was most frequent complication (25%, 11 cases) during 28-day follow-up	Mean peak serum copper levels were higher in cases of AMI with complications ($203.15 \pm 15.13 \mu\text{g}\%$) as compared to cases of AMI without complications ($170.54 \pm 14.18 \mu\text{g}\%$) ($p < 0.001$). AMI with congestive HF had significantly higher serum copper levels as compared to controls
United Kingdom, Kunutsor et al. (2022)	1,866 men aged 42–61 years; 365 HF cases occurred during 26.5 years median follow-up	The HR (95% CI) for incident HF per unit increase in serum copper was 2.71 (1.50–4.89). As serum copper levels increase, the risk of HF gradually increases
Czech Republic, Malek et al. (2006)	30 consecutive subjects with acute decompensation of CHF and 30 patients with chronic stable HF, follow-up of 12 months	The time to the combined end-point death or hospital admission was significantly affected by serum copper level (19.1 vs. $15.6 \mu\text{mol/L}$, $p < 0.0001$). Serum copper levels predicted short term outcome in high risk patients with chronic heart failure
Czech Republic, Málek et al. (2003)	64 patients with CHF; 30 patients died or were admitted to the hospital because of worsening HF during 12 months follow-up	Baseline serum copper concentrations are significantly higher in patients with CHF ($p < 0.001$), also correlating with higher 1-year mortality and morbidity
Iran, Shokrzadeh et al. (2009)	30 ISCMP patients and 27 healthy volunteers	The mean copper level of the ISCMP group ($1.54 \pm 0.52 \text{ mg/L}$) was significantly more than the copper levels of the healthy volunteers ($1.31 \pm 0.24 \text{ mg/L}$; $p = 0.048$). Copper may have a role in the development of ISCMP.
Turkey, Atlihan et al. (1990)	29 patients (mean age 2.3 ± 1.5 years) with CHF and 11 healthy controls (mean age 3.1 ± 2.8 years)	The mean serum copper levels of the patients with CHF showed a significant increase compared to controls ($173.6 \pm 26.6 \mu\text{g}/100 \text{ mL}$ vs. $113.9 \pm 16.2 \mu\text{g}/100 \text{ mL}$, $p \leq 0.001$)
Germany, Oster, (1993)	20 patients (mean age 50.5 ± 7.2 years) diagnosed with dilated cardiomyopathy and 50 healthy controls (mean age 53 ± 8 years)	The patients with dilated cardiomyopathy have high copper concentrations in their serum. The copper concentration in serum shows a highly significant inverse relationship with the ejection fraction and the cardiac index
Turkey, Koşar et al. (2006)	54 HF patients and 30 healthy subjects	Serum copper concentrations in HF patients were significantly higher than in controls ($p = 0.000$)
France, Cenac et al. (1996)	35 patients with peripartum cardiac failure and 40 healthy controls	Plasma copper was higher in patients with peripartum cardiomyopathy than it was in controls (2.03 ± 0.37 vs. $1.23 \pm 0.20 \mu\text{g}/\text{mL}$, $p < 0.001$)
Turkey, Topuzoglu et al. (2022)	54 patients (aged 18–75 years, the left ventricular ejection fraction $<35\%$) with idiopathic dilated cardiomyopathy and 20 healthy subjects (aged 21–73 years)	Patients with idiopathic dilated cardiomyopathy have higher copper (172.16 ± 47.6 vs. $117.28 \pm 31.3 \mu\text{g}/\text{dL}$, $p < 0.05$)
France, de Lorgeril et al. (2001)	21 consecutive CHF patients and 18 healthy age- and sex-matched controls	Plasma copper was slightly higher in CHF than in healthy controls (1.15 ± 0.34 vs. $0.97 \pm 0.17 \text{ mg/L}$, $p < 0.05$)
United State, Sullivan et al. (1979)	42 patients with arteriosclerotic congestive heart failure and 37 healthy controls	Copper levels were elevated in congestive heart failure (1.38 ± 0.33 vs. $1.06 \pm 0.3 \mu\text{g}/\text{mL}$, $p < 0.005$)

AHF, acute heart failure; CHF, chronic heart failure; AMI, acute myocardial infarction; ISCMP, ischemic cardiomyopathy.

proteins that sequester intracellular Cu(I). Specifically, excessive intracellular copper is chelated by two main antioxidant peptides: glutathione (GSH) and MTs (Shishido et al., 2001; Aliaga et al., 2016). Additionally, copper is stored in specialized vesicles and secretory granules (Bonnemaison et al., 2009; Leary and Ralle, 2020). Emerging evidence indicates that the thioredoxin system contributes to the regulation of copper-induced oxidative stress, particularly in neuronal cells (Tanaka et al., 2018). Thioredoxin-albumin fusion proteins have been shown to suppress ROS production and downregulate oxidative stress-related gene expression without significantly affecting intracellular copper levels (Tanaka et al., 2018). These findings suggest an antioxidative mechanism independent of direct copper

sequestration, which may have broader implications for mitigating copper-mediated cytotoxicity in other tissues, including cardiac cells.

2.2.4 Copper export

The export of intracellular copper relies on transporting proteins capable of actively removing excessive copper. In humans, ATP7A and ATP7B are critical copper transporters (La Fontaine and Mercer, 2007). When cellular copper levels are increased, these transporters undergo copper-mediated conformational changes and translocate from the TGN to the cell membrane, thereby promoting copper transport (Petris and Mercer, 1999; Yang et al., 2023). This process requires energy from ATP hydrolysis to transport copper

along a concentration gradient (Lutsenko, 2021). Therefore, the activities and trafficking of ATP7A and ATP7B are stringently controlled by intracellular copper concentrations, copper-binding proteins such as MTs, and multiple signaling pathways (La Fontaine and Mercer, 2007; Lutsenko et al., 2007a; Gupta and Lutsenko, 2009).

3 Evidence linking copper dyshomeostasis to HF

Any abnormality or combination of abnormalities that disrupt cardiac structure, mechanics, or electrical function can potentially trigger HF. Conditions such as atherosclerosis, myocardial infarction (MI), cardiomyopathy, hypertension, and valvular heart disease (VHD) are notable contributors (Heidenreich et al., 2022). Among these, ischemic heart diseases have a significant effect on both acute and chronic HF (Arrigo et al., 2020). Research has increasingly linked the dysregulation of copper homeostasis—resulting in either excessive or deficient copper levels—to the development of HF.

Higher copper levels have been extensively suggested to be associated with HF (Table 1). For example, several prospective cohort studies have indicated that increased serum copper levels are significantly linked to increased HF-related mortality (Málek et al., 2003; Malek et al., 2006). An increased serum copper-to-zinc ratio has also been associated with increased HF risk among middle-aged Finnish males (Kunutsor et al., 2022). Additionally, Hammadah et al. reported in a study involving 890 patients who higher levels of CP, which transports over 95% of copper in the body, were related to an increased risk of HF and poor prognostic outcomes (Hammadah et al., 2014). Measuring CP levels in conjunction with N-terminal pro-B-type natriuretic peptide levels is advantageous for identifying high-risk HF patients during a 1-year follow-up (Romuk et al., 2004). Further investigations into the relationship between myocardial and serum copper contents in patients with HF are essential. In some experiments, coronary infusion of CuCl₂ solutions can induce acute cardiac dysfunction, with the effects of Cu(II) infusion occurring within minutes in both diabetic and normal hearts, suggesting that these effects are not due to remodeling (Cheung et al., 2015). These findings suggest that increased copper content may be related to the pathogenic mechanisms underlying HF.

Copper ions reportedly have dual effects on HF. A study in Poland reported serum copper deficiency in patients with HF (in 44% of men and >30% of women). Copper is an essential antioxidant nutrient for cardiovascular health (Mohammadifard et al., 2019), as evidenced by the benefits of copper supplementation and a copper-rich diet. The Institute of Medicine recommends a daily dietary copper intake of 0.9 mg for adults, with a tolerable upper limit of 10 mg/day to prevent liver toxicity (Trumbo et al., 2001). While national guidelines vary, most suggest a range of 0.8–2.4 mg/day (Bost et al., 2016). Evidence from a high-risk cross-sectional study involving 1,197 healthy adults revealed a negative correlation between dietary or serum copper levels and total/low-density lipoprotein cholesterol, suggesting that increased copper intake is associated with improved metabolic outcomes (Bo et al., 2008). Similarly, copper supplementation

was shown to partially reverse pathological changes caused by dietary iron overload in mice, normalize cardiac hypertrophy (Wang et al., 2018), and enhance cardiac function in pressure overload-induced dilated cardiomyopathy (Hughes et al., 2008). HF patients often exhibit relative deficiencies in multiple micronutrients due to decreased intake, increased metabolic degradation, and excessive excretion, all of which exacerbate cardiac dysfunction (Witte and Clark, 2002). Supplementation with copper-containing micronutrients has been shown to improve the left ventricular ejection fraction, ventricular volume, and quality of life in elderly patients with left ventricular systolic dysfunction and HF (Witte et al., 2005). Cohort studies further suggest that dietary copper intake exceeding the estimated average requirement is associated with reduced cardiovascular and all-cause mortality, particularly when copper is obtained from food sources (Chen et al., 2004). Additionally, more than 80 anatomical, biochemical, and physiological parallels have been identified between copper-deficient animals and patients with ischemic heart disease (Klevay, 2006). In murine studies, dietary copper deficiency impaired β -adrenergic responsiveness and induced diastolic dysfunction, implicating copper insufficiency as a contributor to HF. These impairments were reversed with adequate copper consumption (Elsherif et al., 2003; Elsherif et al., 2004b; Liu and Miao, 2022). Collectively, these findings suggest that dietary copper supplementation may represent a cost-effective therapeutic strategy for managing HF.

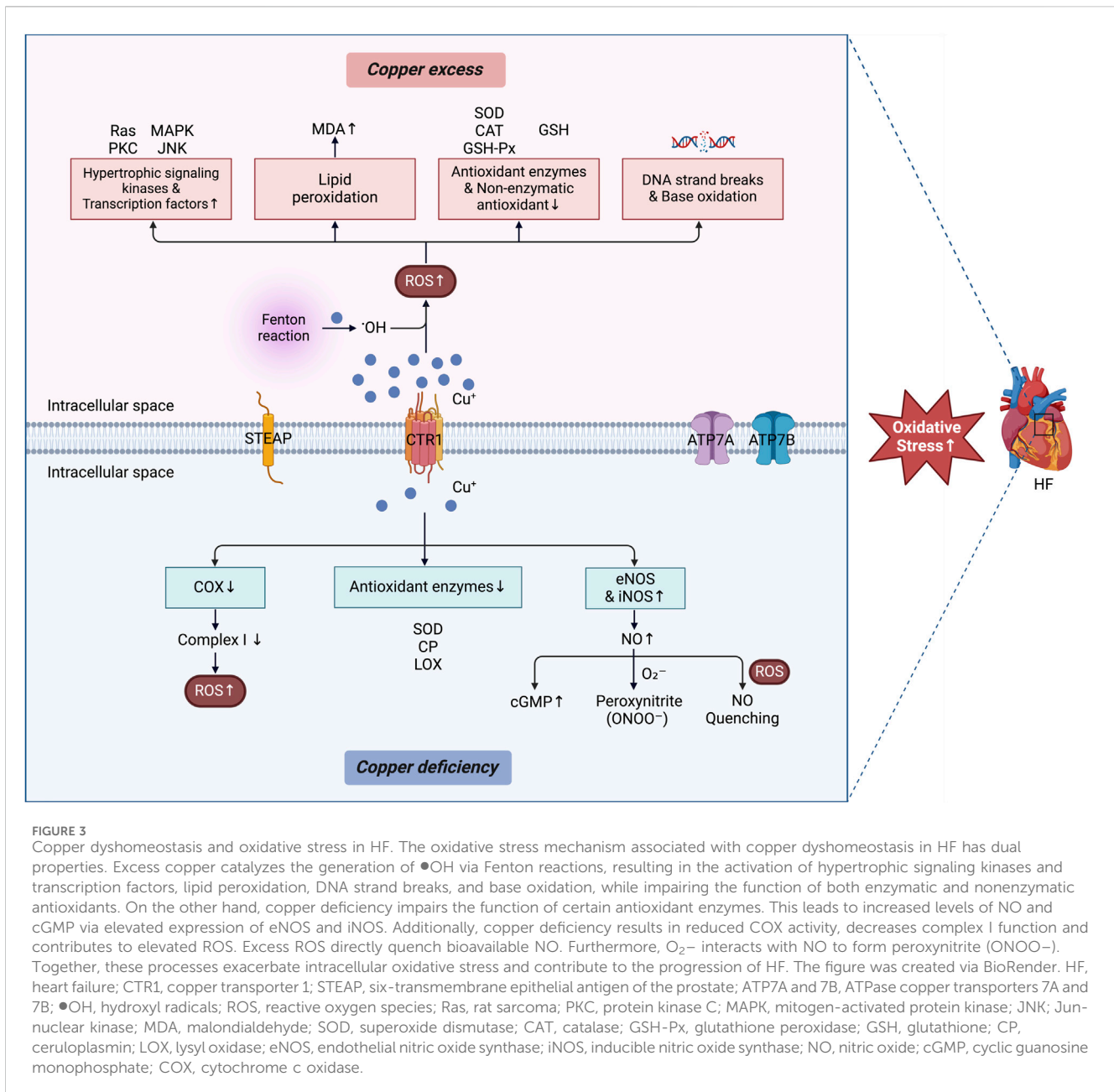
Overall, the role of copper supplementation in the cardiovascular system remains unclear. Other studies have reported that serum copper levels do not significantly differ between patients with HF and control individuals (Cunha et al., 2002; Salehifar et al., 2008; Ghaemian et al., 2011). These inconsistent findings are likely associated with variations in study designs, the duration of copper supplementation, and the dosages used.

4 Potential mechanisms of HF associated with copper dyshomeostasis

Copper is involved in enzymatic activities, mitochondrial respiration, the maintenance of protein function, and iron metabolism (van den Berghe and Klomp, 2009; Dupuy et al., 2015; Niu et al., 2020; Guo et al., 2022b). The prevalence of copper excess in the human population actually surpasses that of copper deficiency, which is partly attributed to high inorganic copper ion consumption via mineral and vitamin supplements, as well as in drinking water from copper plumbing (Brewer et al., 2010; Pal et al., 2014). Hence, copper-mediated HF may be associated with several mechanisms described in this review.

4.1 Oxidative stress

Oxidative stress plays a significant role in the occurrence and development of HF, typically resulting from an imbalance between ROS production and antioxidant defense mechanisms (Karabacak et al., 2014). Copper, a transition metal involved in redox reactions,



facilitates ROS production, with prolonged exposure leading to oxidative stress (Vo et al., 2020) (Figure 3).

Higher levels of free copper ions may further interact with hydrogen peroxide through Fenton reactions, resulting in the generation of highly reactive hydroxyl radicals (Valko et al., 2005). These radicals induce cellular dysfunction, DNA damage, and lipid and protein peroxidation, ultimately promoting the initiation and progression (Tsutsui et al., 2011) of HF. Specifically, copper can activate various hypertrophic signaling kinases and transcription factors, including protein kinase C, GTP-binding protein Rat sarcoma, Jun N-terminal kinase, and mitogen-activated protein kinases (MAPK) (Grubman and White, 2014). This activation stimulates myocardial development, contributes to cellular dysfunction, and facilitates matrix remodeling. Lipid peroxidation is a chain reaction triggered by

the accumulation of ROS in polyunsaturated fatty acids within cell membrane lipids, thereby resulting in lipid molecule oxidative damage. As an intracellular copper scavenger, MT has been demonstrated to be an endogenously expressed and highly inducible antioxidant protein in the heart. Yin et al. reported that mice with silenced MT1/2 genes developed severe HF, cardiac fibrosis, and oxidative stress, with these symptoms exacerbated by intermittent hypoxia. In contrast, mice exhibiting cardiac-specific overexpression of MT-IIa were protected from cardiomyopathy induced by intermittent hypoxia. This protective effect is associated with reduced cardiac lipid peroxidation in the context of copper deficiency (Yin et al., 2014). Furthermore, increased copper levels may increase lipid peroxidation, leading to increased generation of malondialdehyde (MDA) (Dhalla et al., 2000), whose high levels are associated with HF (Díaz-Vélez et al.,

1996; Ghatak et al., 1996; Sairam et al., 2017). Additionally, increased copper levels can decrease the activities of antioxidant enzymes, including SOD, total antioxidant capacity, GSH peroxidase (GSH-Px), and catalase, in both serum and heart tissue while also lowering the levels of the nonenzymatic antioxidant GSH (Skrajnowska et al., 2013; Li et al., 2018). Blood GSH levels serve as an independent marker of lipid peroxidation in HF (Campolo et al., 2023). Copper can induce DNA strand breaks and base oxidation through free radicals generated from oxygen (Brezová et al., 2007). Existing evidence suggests that copper-induced oxidative stress significantly impacts HF.

4.2 Inflammation

Excessive copper is been related to the pathogenic mechanisms of HF because it can cause inflammation. Since its first discovery by Levine et al. (1990), higher circulating levels of proinflammatory factors have been associated with poor HF outcomes (Aukrust et al., 2002; Heymans et al., 2009; Hansson and Hermansson, 2011). Reportedly, increased copper levels can stimulate increases in the serum levels of tumor necrosis factor α (TNF- α) and C-reactive protein in rats, contributing to inflammatory damage in multiple organs, including the heart, and inducing myocardial fibrosis (Wang et al., 2022). *In vitro* experiments revealed that copper enhances interleukin (IL)-6 release while activating MAPK extracellular signal-regulated kinase 1/2 and p38 in primary cardiac cells (Ansteinsson et al., 2009), which are associated with mechanisms of cardiac hypertrophy, an important risk factor for the development of HF (Bueno and Molkentin, 2002; Wenzel et al., 2005). Copper-mediated oxidative stress further exacerbates inflammation, as excess copper leads to excessive ROS accumulation, which then increases myeloperoxidase activity, activates the nuclear factor- κ B (NF- κ B) pathway, inhibits anti-inflammatory cytokines, and leads to a proinflammatory environment (Pereira et al., 2016; Jian et al., 2020).

Additionally, the molecular interplay between copper homeostasis and immune signaling pathways has revealed novel mechanisms by which copper drives excessive and deleterious inflammation during disease progression. Copper uptake, which is mediated by CD44, regulates immune cell activation; however, dysregulated activation triggers uncontrolled inflammation, leading to tissue damage and organ failure, with copper directly acting as a catalytic metal (Solier et al., 2023). Furthermore, copper has been shown to activate the innate immune pathway through ALPK1 in a kinase-dependent manner, amplifying downstream signaling and increasing proinflammatory cytokine production (Lu et al., 2024). Although autoimmunity is not a predominant driver of cardiac dysfunction, it remains a potential target for HF prevention (Martini et al., 2024).

4.3 Energy metabolism dysfunction

Patients with HF typically exhibit features of inadequate cardiac energy metabolism, primarily because of reduced mitochondrial oxidative capacity (Lopaschuk et al., 2021). Copper serves as a

cofactor for several enzymes involved in the antioxidant system and the mitochondrial respiratory chain (Nair and Mason, 1967). However, copper accumulation negatively affects mitochondrial activity and may contribute to HF progression by reducing mitochondrial metabolism (Fan et al., 2005; Rines and Ardehali, 2013). For example, increased copper levels can lead to excessive ROS generation, which facilitates lipid peroxidation, depletes antioxidants, damages mitochondrial DNA, and reduces mitochondrial ATP production (Sugamura and Keaney, 2011; Tsutsui et al., 2011). Furthermore, the failing heart also exhibits a marked decrease in key enzymatic activities, such as mitochondrial creatine kinase (CKmito), which operates at the intersection of energy metabolism and oxidative stress (Keceli et al., 2022). CKmito dysfunction disrupts efficient energy transfer between mitochondria and myofibrils, exacerbating energy deprivation in cardiomyocytes (Keceli et al., 2022). Mitochondrial ATP is primarily generated through fatty acid oxidation, which serves as the heart's main fuel source (Yamamoto and Sano, 2022). Copper can also directly impair enzymes associated with the mitochondrial oxidative phosphorylation (OXPHOS) chain and fatty acid β -oxidation, resulting in mitochondrial insufficiency and energy starvation (Mazi et al., 2020). This cascade of events ultimately contributes to HF progression.

4.4 Cell death

High copper levels are associated with cell death, such as pyroptosis, apoptosis, ferroptosis, and autophagy (Jiang et al., 2022).

4.4.1 Apoptosis

Apoptosis is a programmed cell death process that plays a crucial role in the progression of HF (Vanempel et al., 2005). Copper can induce apoptosis primarily by causing DNA damage, generating ROS, and suppressing proteasome activity (Sagripanti et al., 1991; Kawakami et al., 2008; Chen X. et al., 2021). The apoptosis of myocardial cells, which are the contractile units of myocardial tissue, triggers various reactions, such as fibrosis and hypertrophy. These reactions ultimately lead to both contractile and diastolic dysfunction, contributing to the progression of HF (Vanempel et al., 2005).

4.4.2 Pyroptosis

Pyroptosis is a proinflammatory form of programmed cell death resulting from the activation of caspase family proteins (Kovacs and Miao, 2017; Wu et al., 2021). The increased cellular uptake of copper, combined with increased Fenton activity, can significantly increase intracellular ROS levels, thereby activating Caspase-1 proteins and pyroptosis-related genes, such as nucleotide-binding oligomerization domain-like receptor protein 3 inflammasomes and IL-1 β . This activation subsequently leads to gasdermin D cleavage, ultimately inducing pyroptosis (Jiang K. et al., 2007; Liao et al., 2019; Zhang et al., 2021). Moreover, copper ions, as essential trace nutrients involved in various physiological processes, can induce ROS and activate the NF- κ B pathway. This process upregulates the expression of proinflammatory factors and genes, which may contribute to the development of HF (Butts et al., 2015; Liu et al., 2020; Deng et al., 2023).

4.4.3 Autophagy

Copper ions can trigger autophagy (Tsang et al., 2020). Mechanistically, increased copper levels produce excessive ROS during oxidation, which is associated with the occurrence of autophagy (Li et al., 2015). Additionally, excessive copper may increase autophagic flux through the activation of the Beclin-1 (Wan et al., 2020), autophagy-related gene 5 (Wan et al., 2020), and the adenosine monophosphate-activated protein kinase-mammalian target of rapamycin (Guo et al., 2022a) pathways, promoting the generation of autophagic vesicles by regulating the transcription factor EB (Peña and Kiselyov, 2015). Autophagy has dual functions. Excessive mitophagy and autophagy in various HF models are recognized as protective responses of cardiomyocytes (Shires and Gustafsson, 2015). However, some researchers have suggested that impaired mitophagy can lead to unfavorable myocardial pathological remodeling. The activation of mitochondrial autophagy can cause the heart to transition from adaptive compensatory hypertrophy to myocardial fibrosis, ultimately progressing to HF (Zhu et al., 2007; Li A. et al., 2022). These outcomes may be influenced by the strength of the stimulus and the type of substrate targeted for degradation (Xue et al., 2023a).

4.4.4 Ferroptosis

Ferroptosis refers to iron-dependent cell death resulting from ROS deposition and lipid peroxidation (Dixon et al., 2012; Yang and Stockwell, 2016; Hirschhorn and Stockwell, 2019) and is affected by copper content because copper ions are redox active (Ren et al., 2021; Zhou et al., 2023). GSH-Px 4 (GPX4) is a crucial gene associated with ferroptosis. Copper can directly bind to the GPX4 protein, leading to the formation of GPX4 aggregates and subsequent autophagic degradation of GPX4, ultimately resulting in ferroptosis (Xue et al., 2023b). Copper chelators may reduce sensitivity to ferroptosis without affecting other types of cell death. Fisetin has cardioprotective effects; it modulates GPX4 expression to increase its antioxidant activity and suppress ferroptosis, ultimately ameliorating cardiac hypertrophy (Li et al., 2016).

Additionally, agents that bind to copper, along with their corresponding copper complexes, such as elesclomol-copper and disulfiram-copper, can disrupt mitochondrial homeostasis and induce oxidative stress, ultimately leading to ferroptosis in cancer cells (Wollert et al., 2002; Gao et al., 2021). These findings align with evidence that excess copper deposition generates excessive ROS within cardiomyocytes (Huang et al., 2024).

4.5 Dual effects of copper dyshomeostasis in the induction of HF

Copper deficiency contributes to the occurrence and progression of HF through multiple mechanisms, particularly its impact on oxidative stress. Copper is an essential cofactor for several antioxidant enzymes, including Cu/Zn superoxide dismutase (Cu/Zn SOD), CP, and LOX (Al-Aubaidy et al., 2015). Consequently, copper deficiency can impair the activity of these enzymes, leading to a compromised antioxidant defense system and increased susceptibility to oxidative stress (Chen et al., 1994; Lynch and Colón, 2006; DiNicolantonio et al., 2018). In addition to its

effects on antioxidant enzymes, copper deficiency can decrease COX activity, leading to the oxidative inactivation of complex I (NADH:ubiquinone oxidoreductase). This process can subsequently increase ROS production in copper-deficient cells, thereby exacerbating oxidative stress (Johnson and Thomas, 1999). Copper supplementation has been shown to restore COX activity, resulting in the reversal of myocardial hypertrophy (Jiang Y. et al., 2007). Copper deficiency also disrupts NO homeostasis, particularly by affecting Cu/Zn SOD activity in endothelial cells (Lynch et al., 1997). NO is a crucial vasodilator that plays an essential role in maintaining cardiovascular health. Reduced copper levels lower NO production, leading to endothelial dysfunction, impaired vasodilation, increased oxidative stress, and vascular complications associated with HF (Al-Aubaidy et al., 2015). Interestingly, copper deficiency has a different effect on NO generation in the heart than in endothelial cells. For example, Sarri et al. reported that copper deficiency promoted NO production in the rat heart by increasing the expression of inducible NO synthase and endothelial NO synthase proteins (Saari and Dahlen, 1998; Saari, 2000; Saari et al., 2007). While NO can facilitate HF through its interaction with superoxide to form peroxynitrite, a reactive and long-lived radical that amplifies oxidative stress (Kim et al., 1999; Wollert and Drexler, 2002), excess ROS can also directly quench bioavailable NO. As noted by Paolocci et al., this reduction in NO bioavailability impairs its vasorelaxant capacity and contributes to endothelial dysfunction (Paolocci et al., 2001). The balance between these two effects—NO consumption through peroxynitrite formation and reduced NO availability due to ROS quenching—underpins the endothelial dysfunction and vascular complications observed in HF in the context of copper deficiency. Furthermore, copper deficiency increases cyclic guanosine monophosphate levels, which can impair cardiac contractility and exacerbate HF progression in animal models (Saari and Dahlen, 1998; Saari, 2000; Saari et al., 2007). Collectively, these findings underscore the complex role of NO in the pathophysiology of heart disease associated with copper deficiency. However, further studies are needed to elucidate the molecular mechanisms by which NO regulates cardiac pathologies in the context of copper deficiency.

Decreased responsiveness to β -adrenergic stimulation is a hallmark of copper deficiency-induced HF, as evidenced by the reduced sensitivity of copper-deficient mouse hearts to the β -adrenergic agonist isoproterenol (Elsherif et al., 2003). β -Adrenergic receptors, members of the G-protein-coupled receptor family, are regulated through changes in expression and function in response to external stimuli, resulting in alterations in heart rate, contractility, relaxation, and automaticity (Pfleger et al., 2019). Dysregulation of β -adrenergic receptor signaling has been associated with congestive heart failure caused by cardiomyopathy. Proposed mechanisms include reduced receptor expression, downregulation of G-proteins, or impaired adenylate cyclase activity due to phosphorylation (Elsherif et al., 2003). The impaired adrenergic responsiveness observed in copper-deficient hearts raises questions about its potential relationship with altered catecholamine metabolism under copper-deficient conditions (Gross and Prohaska, 1990; Prohaska et al., 1990; Seidel et al., 1991). Notably, functional changes have been shown to precede structural damage in copper deficiency. In rats subjected to a

copper-restricted diet for 9 or 15 months, both diastolic and systolic dysfunctions were observed. This was indicated by a blunted response in the maximal left ventricular pressure elevation rate (+dP/dt), the maximal left ventricular pressure decrease rate (-dP/dt), and the left ventricular end-diastolic pressure in response to isoproterenol (Li et al., 2005). In a previous study, feeding copper-deficient mice a diet adequate in copper for 4 weeks completely restored cardiac systolic and diastolic functions, as well as responsiveness to β -adrenergic stimulation (Elsherif et al., 2004b). These findings indicate that the response of the heart to β -adrenergic stimulation is dependent on copper levels.

The molecular mechanisms underlying HF induced by copper deficiency involve disrupted cellular calcium homeostasis. Altered myocardial contractility during end-stage HF is associated with changes in Ca^{2+} cycling (Hasenfuss et al., 1996; Hasenfuss and Pieske, 2002). This homeostasis is regulated primarily by sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase (SERCA), ryanodine receptors (RyRs), and the sodium/calcium exchanger (NCX) (Bers, 2002). Kang et al. reported that a copper-deficient diet markedly altered the expression of calcium cycling genes in the mouse heart, including a reduction in L-type calcium channels, which affected calcium release from the sarcoplasmic reticulum via potassium-dependent NCX and RyRs. Although there is a lack of cardiac functional data, the expression of these calcium-regulating genes was notably normalized in mice with copper deficiency following supplementation with a copper-replete diet (Elsherif et al., 2004a). Additionally, copper deficiency may impair calcium homeostasis and cardiac contractile activity by increasing phospholamban levels, which inhibits SERCA2a-mediated calcium uptake (Elsherif et al., 2007).

Additionally, copper deficiency has been reported to lead to alterations in myocardial gene expression in mice, particularly in genes involved in cardiac contractility, fibrosis, and inflammation. These changes are potential factors contributing to the alterations in cardiac activity observed in mice with copper deficiency (Elsherif et al., 2007). Further studies are essential to explore the underlying mechanisms of these findings.

5 Copper-mediated cell death and HF

5.1 Copper-mediated cell death and cuproptosis

Copper-mediated cell death was first discovered in the late 1970s when Chan et al. identified mechanisms regulating copper levels within healthy fibroblasts, noting that increased copper levels resulted in cell death (Chan et al., 1978). However, the precise mechanisms remain unknown. Since then, the copper-mediated cell death mechanism has garnered significant attention from researchers, and copper has been found to have dual effects. Over the last decade, the toxicity of essential trace metals to mammalian cells has become increasingly understood. In a recent study, such metals were found to induce cell death through mechanisms independent of established pathways, such as apoptosis or necrosis, as observed with zinc and iron (Dineley et al., 2003; Kagan et al., 2017; Du et al., 2021). This observation may also signal the gradual emergence of a mechanism for a noncanonical copper-induced cell death pathway.

In March 2022, Tsvetkov et al. published a groundbreaking paper in *Science* unveiling cuproptosis, a unique form of regulated cell death resulting from mitochondrial copper accumulation (Tsvetkov et al., 2022); they reported that copper accumulation in cells was a major factor triggering cuproptosis. Notably, after copper ionophores induce cell death, traditional markers of cell death, such as caspase-3, are not detected. Furthermore, only copper chelators can rescue cells from elesclomol-induced death. Inhibiting established cell death pathways—such as apoptosis, necroptosis, oxidative stress, and ferroptosis—fails to prevent cell death, highlighting the distinct nature of cuproptosis.

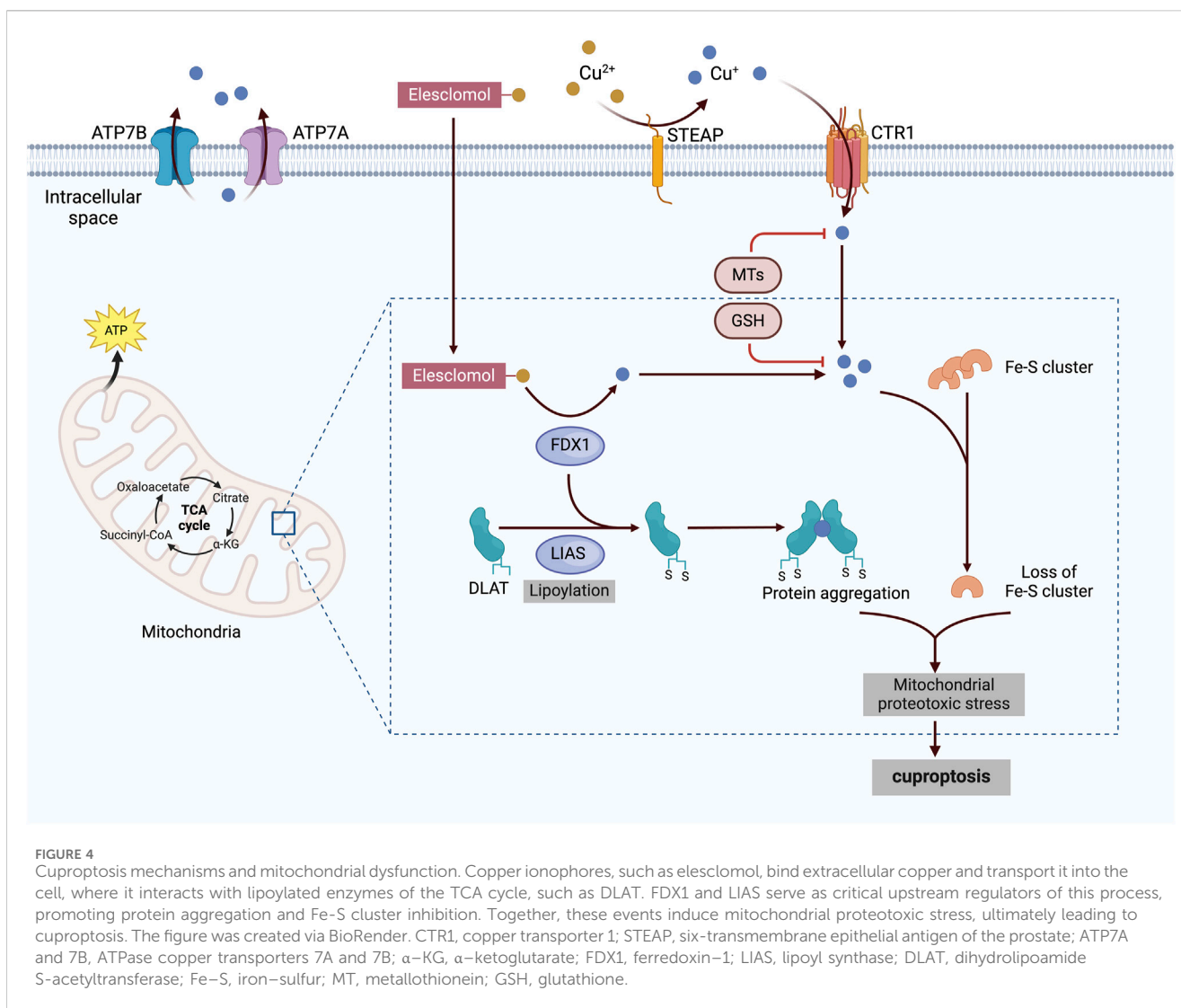
Tsvetkov et al. reported that copper ionophore-induced cell death is dependent on mitochondrial respiration, as evidenced by the heightened sensitivity of mitochondria-dependent cells to copper ionophores, which are 1,000 times more sensitive than glycolytic cells (Tsvetkov et al., 2022). Further investigations revealed a time-dependent increase of tricarboxylic acid (TCA) cycle metabolite levels in cells exposed to copper ionophores, underscoring the close relationship between cuproptosis and the TCA cycle (Li Y. et al., 2022). In cuproptosis, copper in cells can bind to lipoylated components of the TCA cycle, resulting in the aggregation of copper-bound lipoylated mitochondrial proteins. This aggregation disrupts the TCA cycle, thereby impairing cellular energy generation. Ferredoxin and lipoyl synthase, two upstream regulatory factors, play significant roles in this process and have been identified as key contributors to copper toxicity through whole-genome clustered regularly interspaced short palindromic repeat selection (Dreishpoon et al., 2023). The aggregation of proteins and the subsequent decrease in iron-sulfur clusters—important cofactors for various cellular processes, such as enzymatic reactions and electron transport (Lill and Freibert, 2020)—promote toxic protein stress, ultimately leading to cell death (Figure 4).

5.2 Cuproptosis-associated mitochondrial dysfunction and HF

Mitochondria serve as significant reservoirs of intracellular copper, which is a critical component that regulates mitochondrial function. Hence, pathologies and diseases resulting from copper metabolism disorders are closely linked to mitochondrial metabolism (Tian et al., 2023). Mitochondria are highly vulnerable to copper-mediated injury, leading to oxidative damage to their membranes (Arciello et al., 2005; Cobine et al., 2021).

Copper serves as a cofactor for various mitochondrial enzymes involved in ATP production, a process that relies on the TCA cycle and OXPHOS within mitochondria (Arnold and Finley, 2023). However, increased copper content in cells can impair mitochondrial activity by altering critical enzymes associated with these processes (Sheline and Choi, 2004; Wang Z. et al., 2023). This disruption also generates ROS, which subsequently damage the inner mitochondrial membrane, impair the electron transport chain, and compromise mitochondrial DNA. This damage leads to mitochondrial dysfunction and reduced ATP production, ultimately accelerating cell death (Tsutsui et al., 2011).

Furthermore, metabolic enzymes may undergo lipoylation, a conserved posttranslational modification (Rowland et al., 2018).



Lipoylation has been identified in glycine cleavage system protein H, dihydrolipoamide branched-chain transacylase E2, dihydrolipoamide S-acetyltransferase, and dihydrolipoamide S-succinyltransferase, all of which are associated with metabolic complexes that regulate the entry points of carbon into the TCA cycle (Rowland et al., 2018; Solmonson and DeBerardinis, 2018). Lipoylation involves the attachment of lipoic acid, a small sulfur-containing metabolite, to substrate proteins. Notably, lipoic acid can bind copper, leading to the toxic accumulation of lipoylated mitochondrial enzymes within the mitochondria (Kahlson and Dixon, 2022).

Moreover, excessive copper concentrations within the mitochondria can compromise mitochondrial membrane integrity by disrupting the membrane potential and increasing membrane permeability (Gyulkhandanyan et al., 2003; Reddy et al., 2008a). The opening of transmembrane pores, a critical factor in mitochondrial permeability transition, is not fully understood in terms of its precise chemical nature, but it is likely associated with the release of necrotic or apoptotic factors (Zazueta et al., 1998; Su et al., 2011). Additionally, the strong oxidizing nature of Cu(I) further contributes to potentially irreversible damage to the mitochondrial membrane (Reddy et al., 2008b).

The heart is the central organ for energy production in the human body, with about 95% of its ATP generated through oxidative metabolism in the mitochondria (Zhou and Tian, 2018). Consequently, mitochondrial dysfunction is linked to the onset and progression of various cardiovascular diseases. Mitochondrial dysfunction has been reported to affect cardiac energy supply, inflammatory mechanisms, calcium modulation, oxidative stress, and cell death, all of which are critical therapeutic targets for HF (Hammadah et al., 2014; Zhang et al., 2020; Yuan et al., 2022). In studies involving rats with HF, copper chelation and restoration within cardiomyocytes have been shown to repair mitochondria while improving cardiac function (Zhang et al., 2020). Hence, cuproptosis represents a novel mechanism for treating HF.

6 Possible treatments for cuproptosis in HF

Copper-based drugs have been extensively studied not only for cancer treatment but also for their multifaceted impact on HF therapy (Chen et al., 2011; Wang et al., 2024). These agents have

promising effects in the treatment of HF from various perspectives. Copper-based therapies may enhance HF management by providing individualized, targeted, and efficient treatment methods.

6.1 Copper chelators

As a chelator, triethylenetetramine (TETA) specifically and selectively binds to Cu(II) and is used as a second-line therapy for Wilson's disease (Walshe, 1982; Cooper, 2011). TETA treatment can enhance the regeneration of cardiac structure and function in HF model mice with diabetes (Cooper et al., 2004; Zhang et al., 2007; 2014; Nurchi et al., 2016) and reduce pathological left ventricular hypertrophy in patients with diabetes (Cooper et al., 2004; 2009). Moreover, TETA can restore cardiac pump activity by enhancing the activities of mitochondrial proteins such as COX, mitochondrial CCS, and SOD1, as well as by reinstating the expression of peroxisome proliferator-activated receptor gamma coactivator-1 α , an important regulatory factor for mitochondrial biogenesis (Zhang et al., 2020). TETA has reportedly advanced to drug development for treating HF (Cooper G, 2012).

Owing to its copper-chelating properties, tetrathiomolybdate (TTM), which was initially developed for treating Wilson's disease, has been explored for its potential therapeutic benefits in HF (Pufahl et al., 1997; Alvarez et al., 2010), which effectively prevents copper transport and its subsequent incorporation into cuproproteins. Although the effects of TTM on cardiac conditions remain elusive, some studies have highlighted its ability to reduce systemic copper overload and its associated proinflammatory consequences, which are relevant in the context of HF. For example, Wei et al. reported that TTM suppresses the expression of NF- κ B, TNF- α , and monocyte chemoattractant protein-1 in the aorta and heart (Wei et al., 2011), which play crucial roles in inflammation. These findings open avenues for exploring the role of TTM in HF treatment, indicating the need for further research to directly assess its efficacy in treating cardiac diseases.

6.2 Small-molecule inhibitors of copper chaperone proteins

Copper ion chelation therapies can reduce copper levels; however, they lead to various side effects and disrupt various copper-dependent physiological processes (Chen et al., 2011). On the other hand, while copper supplementation can address copper deficiency, it carries the risk of copper overload. Excessive copper levels can exacerbate oxidative stress through the generation of ROS, impair mitochondrial function, and disrupt cellular homeostasis, particularly in the heart and liver (Kaplan and Maryon, 2016). These challenges highlight the need for more targeted approaches to modulate copper homeostasis in HF therapy. To minimize these adverse effects, small-molecule inhibitors of copper chaperone proteins offer a promising alternative. Unlike chelation or supplementation strategies, these inhibitors focus on the selective redistribution and regulation of intracellular copper levels, thereby reducing the risks associated with copper imbalance.

DCAC50 is a promising small-molecule inhibitor that selectively disrupts the functions of copper chaperones by binding to proteins such as CCS and ATOX1 (Karginova et al., 2019). ATOX1 is

involved in transporting copper to the cytosol, whereas CCS facilitates the delivery of copper to SOD1 (Rosenzweig and O'Halloran, 2000). In studies of atherosclerosis, ATOX1 is increased in the intima of atherosclerotic lesions in ApoE^{-/-} mice and is localized to the nucleus under pathological conditions, including hypertensive and atherosclerotic vessels (Jeney et al., 2005; Itoh et al., 2008). Similarly, CCS plays a vital role in angiogenesis and wound healing, with its ability to impair these processes and contribute to the progression of cardiovascular diseases (Fukai et al., 2018). While both ATOX1 and CCS are critical for normal physiology, their dysregulation under pathological conditions underscores the potential for selectively targeting copper chaperones as a therapeutic strategy. Given the pivotal role of copper homeostasis in maintaining cardiac function, DCAC50 offers a targeted approach to modulate copper chaperone activity while preserving physiological copper-dependent processes. Further studies on small-molecule inhibitors of copper chaperone proteins are needed to develop effective treatments for HF.

6.3 Copper ionophores

Copper ionophores represent another therapeutic strategy for modulating copper levels and influencing related cellular pathways in HF. Unlike copper chelators, which remove copper from cells, copper ionophores facilitate the delivery of copper into cells, thereby increasing the intracellular copper concentration. Some examples of these ionophores include pyrithione, disulfiram, elesclomol, and chloroquine (Xue et al., 2023a). Among them, elesclomol has garnered considerable attention owing to its sensitivity to tumor cells and its application in clinical studies for cancer therapy (O'Day et al., 2013). However, the mechanisms underlying its selectivity remain unelucidated, warranting further investigation to determine whether this selectivity can be adapted for treating HF with additional copper ionophores. Su et al. incorporated metal supplements via a targeted ion carrier approach designed to deliver metals to specific sites within the body (Su et al., 2018). This strategy addresses the limitations of conventional copper ionophores, particularly their multifunctionality and lack of specificity, thereby opening new avenues for applying copper in HF treatment. Additionally, nanomedicine-based drug delivery systems are being widely explored to enable the precise delivery of therapeutic agents (Su et al., 2018). These developments underscore the need for future research focused on developing more selective and targeted copper ionophores for HF therapy.

7 Conclusion and future perspectives

Overall, the diverse effects of copper on HF are linked to complex systemic and cellular metabolism. Copper has dual effects on HF, and excessive or deficient copper can contribute to disease progression by regulating oxidative stress, inflammation, energy metabolism, cell death, the response to β -adrenergic stimulation, and calcium homeostasis. Further investigations are warranted to elucidate the interactions among these factors and their effects on HF progression.

Copper-mediated cell death and the subsequent process of cuproptosis can increase our understanding of the effects of

copper on HF. The relationship between cuproptosis and mitochondrial dysfunction in HF underscores the necessity of exploring the molecular mechanisms associated with these processes. The development of copper-based therapies that target cuproptosis is a promising approach and has therapeutic potential for the treatment of not only cancer but also cardiovascular diseases. However, further investigations are needed to determine the role of cuproptosis in causing cell injury and to identify reliable specific biomarkers, which can provide crucial insights for the prevention and management of HF.

Author contributions

ZL: Formal Analysis, Funding acquisition, Visualization, Writing—original draft, Writing—review and editing. YG: Formal Analysis, Visualization, Writing—original draft. ZS: Investigation, Funding acquisition, Visualization, Writing—review and editing. SC: Formal Analysis, Writing—review and editing. XW: Funding acquisition, Investigation, Writing—review and editing. YL: Conceptualization, Writing—review and editing. XL: Conceptualization, Writing—review and editing. HF: Project administration, Writing—review and editing. JC: Funding acquisition, Visualization, Writing—review and editing. NL: Visualization, Writing—review and editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This review

received funding from the National Natural Science Foundation of China (No. 82405146), the Shandong Province Medical and Health Technology Project (No. 202303011361), the Shandong Province Traditional Chinese Medicine Technology Project (No. Q-2023006), the Doctoral Research Start-up Fund of Shandong Second Medical University (No. 04118601 and 04118701) and the Weifang Science and Technology Development Plan Project (No. 2021GX058).

Conflict of interest

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OPEN ACCESS

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RECEIVED 09 January 2025

ACCEPTED 23 May 2025

PUBLISHED 04 June 2025

CITATION

Xiong Y, Xin H, Shi C, Guo X, Chen Y, Huang C, Ma F, Yang G and Yang J (2025) Real-world data of Azvudine-induced hepatotoxicity among hospitalized COVID-19 patients in China: a retrospective case-control study. *Front. Pharmacol.* 16:1558054. doi: 10.3389/fphar.2025.1558054

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Real-world data of Azvudine-induced hepatotoxicity among hospitalized COVID-19 patients in China: a retrospective case-control study

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Background: The COVID-19 pandemic, caused by the SARS-CoV-2 virus, has led to global health crisis. Although several antiviral drugs have been used to mitigate the severity and mortality of COVID-19, the safety profile remained a critical concern. Azvudine, a new nucleoside analog, has been approved for emergency use in China for COVID-19. However, the incidence and risk factors associated with Azvudine-induced hepatotoxicity in hospitalized patients remained unclear.

Objects: To assess the prevalence, risk factors, clinical patterns, and outcomes of Azvudine-induced hepatotoxicity by real-world data.

Methods: We conducted a single-center retrospective case-control study at Renmin Hospital of Wuhan University, including patients administered Azvudine for COVID-19 treatment between December 2022 and May 2023. Univariate and multivariate logistic regression analyses were preformed to assess risk factors for Azvudine-associated or -induced hepatotoxicity. Receiver operating characteristic (ROC) curve analysis was performed to calculate the area under the ROC curve (AUC).

Results: In total, 669 patients were included in the Azvudine-associated hepatotoxicity research. 47.1% patients exhibited hepatotoxicity, abnormal liver function on admission [OR: 5.55 (3.94–7.90), $P < 0.001$] and antithrombotic drugs [OR: 1.79 (1.27–2.54), $P = 0.001$] were independent predictors of Azvudine-associated hepatotoxicity, with the area under the ROC curve (AUC) was 0.756 [95% CI: 0.719–0.792, $P < 0.001$]. Further studies of Azvudine-induced hepatotoxicity revealed 294 cases, of which 27.2% showed hepatotoxicity. The concomitant use of antivirals [OR: 3.80 (1.47–10.1), $P = 0.006$] and anticoagulant drugs [OR: 3.12 (1.77–5.61), $P < 0.001$], particularly Ganciclovir [OR: 4.11 (1.45–12.2), $P = 0.008$], Low-Molecular-Weight Heparin Calcium [OR: 3.00 (1.69–5.33), $P < 0.001$], and Enoxaparin [OR: 2.68 (0.99–7.10), $P = 0.047$], were significantly associated with an increased risk of hepatotoxicity. Most hepatotoxicity cases were mild, and recovered or improved after drug withdrawal and treatment, whereas severe cases contributed to the progression of the primary disease and increased mortality risk.

Conclusion: Our study provided evidence of the significant association between Azvudine and hepatotoxicity in hospitalized COVID-19 patients. These findings

underscored the importance of monitoring liver function during Azvudine treatment and caution against concomitant use of certain medications. Further research was warranted to elucidate the mechanisms underlying Azvudine-induced hepatotoxicity and optimize clinical management strategies.

KEYWORDS

COVID-19, Azvudine, hepatotoxicity, risk factors, real-world data

1 Introduction

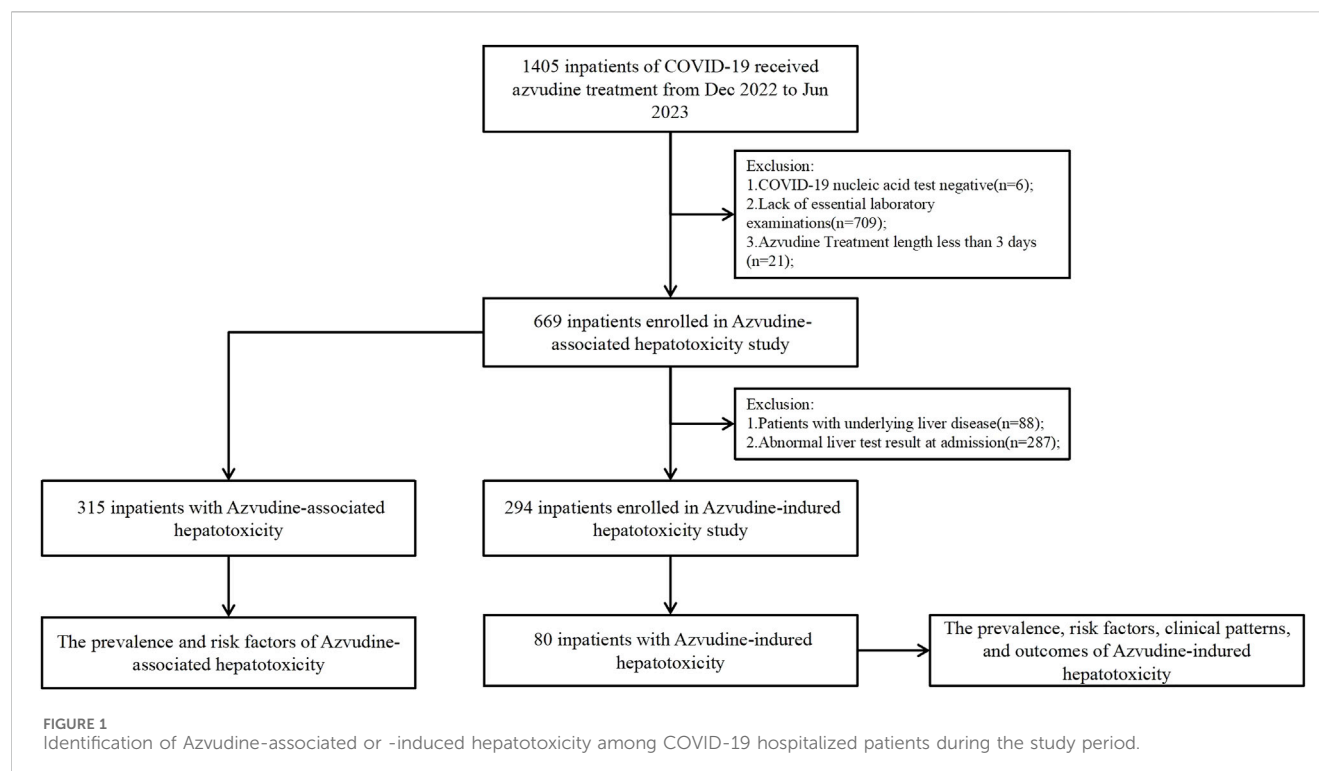
The COVID-19 pandemic, triggered by the SARS-CoV-2 virus, has led to millions of fatalities worldwide, with enduring repercussions on global health systems, economic stability and social structures (Kevadiya et al., 2021; Kwok, 2022; Baden et al., 2021; Rubin, 2021). As of 2024, the COVID-19 pandemic remains a persistent public health challenge, characterized by the emergence of novel variants and sustained international efforts in disease mitigation (Chow et al., 2023; Nanaw J, 2024). To solve the problem, several antiviral drugs, such as Remdesivir, Molnupiravir, and Nirmatrelvir-Ritonavir, have been utilized in the treatment of COVID-19 following to WHO guidelines (Han et al., 2024a; Grein et al., 2020). Additionally, Azvudine (2'-deoxy-2'- β -fluoro-4'-azidocytidine), a novel nucleoside analog, has gained emergency approval for the Omicron variant surge in China (General Office of the National Health Commission, 2022; Deng et al., 2023).

Azvudine is a pro-drug that undergoes intracellular phosphorylation by deoxycytidine kinase phosphorylated in the cytoplasm to form its active metabolite. The active compound exerts antiviral effects primarily through inhibition of the viral RNA-dependent RNA polymerase (RdRp) of viruses (Zhang et al., 2021; Yu and Chang, 2020). Initially developed for HIV treatment, Azvudine has demonstrated favorable efficacy and tolerability profiles in Chinese patients during 48-week therapeutic regimens. Recent studies have revealed its potent activity against SARS-CoV-2 virus with dual mechanisms: effective suppression of viral RNA replication and immunomodulatory effects through thymus-mediated lymphocyte profile enhancement (Ren et al., 2020; Yang et al., 2024a). The pharmacological properties make Azvudine a promising therapeutic candidate for COVID-19. Clinical trials have demonstrated Azvudine significant antiviral efficacy against COVID-19. Prior studies have revealed comparable clinical outcomes between Azvudine and Nirmatrelvir-Ritonavir in hospitalized patients, with no statistically significant differences observed in 28 days all-cause mortality, composite disease progression, and clinical improvement (Han et al., 2024b; Shang et al., 2024). The findings provide robust evidence supporting Azvudine effectiveness as an alternative treatment for COVID-19, with safety and efficacy profile. Compared to Nirmatrelvir-Ritonavir, Azvudine demonstrated comparable clinical efficacy while showing a significantly lower risks of composite disease progression in non-severe COVID-19 patients, indicated Azvudine might offer superior clinical benefits over Nirmatrelvir-Ritonavir in certain patient populations (Yu and Chang, 2022; Wang S. et al., 2024). Further supporting evidence from a retrospective case-control study by Su et al. demonstrated comparable therapeutic efficacy between Azvudine and

Nirmatrelvir-Ritonavir, with no statistically significant differences observed in key clinical outcomes including recovery time, mortality rates, and hospitalization duration (Su et al., 2024). Meta-analysis further indicated that Azvudine may be preferable to Nirmatrelvir-Ritonavir for elderly patients (aged over 75 years old), primarily due to its significantly lower risk of drugs interaction while maintaining comparable efficacy (Wang Y. et al., 2024; Shang et al., 2024). Furthermore, pharmacoeconomic analyses demonstrate that Azvudine exhibited a significantly lower incremental cost-effectiveness ratio (ICER) compare to Nirmatrelvir-Ritonavir (Yang et al., 2024b). The enhanced cost-efficiency profile improved treatment accessibility, particularly in resource-limited and lower-income populations. In summary, current clinical and pharmacoeconomic evidence positions Azvudine as a clinically effective and cost-efficient therapeutic option for COVID-19 management, particularly valuable for resource-constrained healthcare systems.

Previous research has demonstrated that SARS-CoV-2 infection not only affects the respiratory system but also involves multiple organ dysfunction (MODS), thus inevitably leading to the increase in all-cause mortality (Zhong et al., 2020; Alqahtani and Schattner, 2020). Notably, hepatic dysfunction occurs in up to half of all reported cases (Alqahtani and Schattner, 2020; Allison et al., 2023). Medications have been recognized as one of the contributing factor to hepatocellular injury in COVID-19 infections (Zhong et al., 2020; Wen-Shu Hu, 2022; Rodriguez-Espada et al., 2024). Notably, antiviral drugs such as Remdesivir, and Nirmatrelvir-Ritonavir have been associated with an elevated risk of drug-induced hepatotoxicity (Allison et al., 2023; Xie et al., 2020; Naserallah et al., 2022). The safety and tolerability of Azvudine as a novel nucleoside analog has remain critical considerations, particularly in patients with pre-existing liver impairment. According to the manufacturer's label, Azvudine may cause mild-to-moderate liver dysfunction in isolated instances, with approximately 35% of patients exhibiting modest elevation in liver enzyme. Clinical studies have reported hepatotoxicity ranges from 13.5% to 38.4%, udine among COVID-19 patients receiving Azvudine (Lan et al., 2024; Zhi et al., 2023; He et al., 2023; Liu et al., 2024; Yang et al., 2023; Liu S. et al., 2023). However, the generalizability of these findings remains uncertain due to limited sample size. Consequently, further large-scale studies are warranted to clarify the safety profile of Azvudine in COVID-19 treatment.

This study utilized real world data to evaluate the prevalence, risk factors, clinical manifestations, and outcomes of Azvudine-associated hepatotoxicity among in hospitalized COVID-19 patients. The findings aim to establish a comprehensive safety profile of Azvudine and provide evidence-based guidance for clinical decision-making in COVID-19 management.



2 Methods

2.1 Study design and ethics approval

This single-center retrospective case-control study was conducted at the Renmin Hospital of Wuhan University, a tertiary medical center with approximately 7300 beds in China. This study protocol was approved by the Ethics Committee of the Renmin Hospital of Wuhan University (Approval No.: WDRY2024-K003), with waiver of informed consent granted due to the retrospective study.

2.2 Patient inclusion

The diagnosis of COVID-19 has made by infectious disease and internal medicine clinicians based on clinical signs, a chest computed tomography scan, and a positive COVID-19 nucleic acid test result. Patients with respiratory rates ≥ 30 breaths/minute, $SpO_2 \leq 93\%$ on room air, or $PaO_2/FiO_2 \leq 300$ mmHg were categorized as severe cases, while other cases were classified as non-severe (General Office of the National Health Commission, 2022). Patients who were Azvudine for COVID-19 treatment between December 2022 to May 2023 were included. Exclusion criteria for Azvudine associated hepatotoxicity based on the following criteria: 1) Negative COVID-19 nucleic acid test results, 2) Incomplete laboratory data (lack of baseline liver function data within 7 days prior to treatment initiation or absence of follow up, and 3) Azvudine treatment less than 3 days (Shi et al., 2021; Zhao et al., 2024; Lu et al., 2024). Additional exclusion criteria for Azvudine-induced hepatotoxicity included: 1) pre-existing liver disease and 2) abnormal liver function tests at admission.

The patients' comprehensive data including: demographic characteristics, COVID-19 severity, Azvudine dosage, duration of treatment liver disease history, serial liver function tests, comorbidities, and concomitant medications. A schematic flowchart delineating the methodology for identifying Azvudine-associated and Azvudine-induced hepatotoxicity cases was shown in Figure 1.

2.3 Evaluation of hepatotoxicity

Hepatotoxicity was defined as liver function biochemical abnormalities s when meet at least one of the following biochemical criteria: 1) alanine aminotransferase (ALT) > 40 U/L; 2) aspartate transaminase (AST) > 35 U/L; 3) alkaline phosphatase (ALP) > 135 U/L; 4) gamma-glutamyltransferase (GGT) > 45 U/L; 5) total bilirubin (TB) > 23 μ mol/L (Yu et al., 2017). All values represent the upper limit of normal (ULN) for our laboratory reference ranges. In this study, patients who developed hepatotoxicity after Azvudine treatment were categorized into the Abnormal group, while those without hepatotoxicity were classified as the Normal group.

The causality of Azvudine-induced hepatotoxicity was evaluated using the updated Roussel Uclaf Causality Assessment Method (RUCAM) (Danan and Teschke, 2016; Zhao et al., 2021), with cases scoring below 3 on the RUCAM scale being excluded from analysis due to insufficient evidence for establishing causal relationship between liver injury and Azvudine administration. To classify the clinical type of Azvudine-induced hepatotoxicity, the R value was calculated using the following formula: $R = [ALT / (ALT \text{ ULN})] / [ALP / (ALP \text{ ULN})]$. Based on established criteria (Andrade et al., 2019a; Andrade et al., 2019b), drug-induced liver

injury was categorized into three clinical types: 1) hepatocellular injury ($R \geq 5$); 2) cholestatic injury ($R \leq 2$); 3) mixed injury ($2 < R < 5$).

The severity of Azvudine-induced hepatotoxicity was classified according to the biological criteria Common Terminology Criteria for Adverse Events (CTCAE) Version 5.0, based on the ULN thresholds of ALT, ALP, and GGT as follows: 1) Grade 1 (Mild): elevation of ALT or ALT $\geq 1 \times$ ULN and $< 3 \times$ ULN, elevation of ALP or GGT $\geq 1 \times$ ULN and $< 2.5 \times$ ULN; 2) Grade 2 (Moderate): elevation of ALT or ALT $\geq 3 \times$ ULN and $< 5 \times$ ULN and elevation of ALP or GGT $\geq 2.5 \times$ ULN and $< 5 \times$ ULN; 3) Grades 3 and 4 (severe induced liver injury, DILI): elevation of ALT or ALT $\geq 5 \times$ ULN and elevation of ALP or GGT $\geq 5 \times$ ULN.

2.4 Prognosis of azvudine-induced hepatotoxicity

The prognosis of Azvudine-induced hepatotoxicity was categorized based on post-treatment liver function records was as follows: 1) Complete Recovery: liver enzyme levels returned to within normal limits (below ULN) or baseline values; 2) Partial Improvement: ALT/AST decreased to below $3 \times$ ULN or ALP/GGT decreased to below $2.5 \times$ ULN; 3) Aggravation: liver test values exceeded previous peak levels; 4) Unknown: Insufficient follow-up data for proper assessment.

2.5 Statistical analysis

Statistical analysis was conducted using SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA). Continuous variables with normal variables were reported as mean $\pm$ standard deviation (SD), whereas non-normally distributed variables were presented as medians (interquartile range, IQR). Independent sample t-tests and Mann-Whitney U tests were for comparisons. Categorical variables were analyzed using either chi-square test or Fisher's exact test, as appropriate. For multivariable analysis, variables demonstrating a potential association ($P < 0.20$) in univariate analysis were entered into a logistic regression model. Independent risk factors for Azvudine-associated and Azvudine-induced hepatotoxicity were identified through stepwise logistic regression. A two-tailed P value < 0.05 was considered statistically significant for all analysis.

We performed sensitivity analyses to evaluate the robustness and generalizability of primary findings, following established methodological approaches (Hykin et al., 2019; Liu ZQ. et al., 2023). In the current analysis, we mainly conducted sensitivity analyses on two specific subgroups. Age-stratified analysis: We introduced an age-treatment interaction term in the regression model to specifically examine potential effect modification in patients aged more than 35 years old. Allergy adjusted analysis: we systematically exclude patients with documented drug allergy histories and re-analyzed the primary outcomes to assess the potential confounding effect of medication hypersensitivity. These rigorous sensitivity assessments were designed to:

- 1) Verify the consistency of treatment effects across clinically relevant subgroup
- 2) Examine the potential influence of key confounding variables
- 3) Enhance the external validity of the conclusion for diverse patient population.

3 Results

3.1 Patients enrolled

We initially identified 1,405 hospitalized COVID-19 patients through electronic medical record review, we implement the following exclusion criteria: Six patients (0.4%) with negative COVID-19 nucleic acid tests, 709 (50.5%) lacking essential laboratory examinations, and 21 (1.5%) with follow-up durations of less than 3 days were excluded; 669 patients (47.6%) were included in the Azvudine-associated hepatotoxicity research (Figure 1). The enrolled population was subsequently stratified into two groups according to hepatotoxicity status, with comprehensive clinical characteristics presented in Table 1.

To establish a baseline of normal liver function prior to treatment, we implement additional exclusion criteria: 88 (13.2%) cases were excluded due to liver disease, and 287 (42.9%) cases were excluded due to abnormal liver function, the remaining 294 (43.9%) cases enrolled in the study of Azvudine-induced hepatotoxicity (Figure 1). The final cohort was dichotomized based on the development of treatment-emergent liver injury, with comprehensive demographic and clinical characteristics detailed in Table 2.

3.2 Characteristics of COVID-19 patients with Azvudine-associated hepatotoxicity

Our analysis of 669 hospitalized COVID-19 patients revealed that 315 cases (47.1%) exhibited abnormal liver function test, whereas 354 patients (52.9%) maintained normal hepatic parameters, as shown in Table 1. Multivariate logistic regression identified two significant risk factors for hepatotoxicity. (1) male gender (OR 1.63, 95% CI 1.18–2.27, $P = 0.003$), and (2) severe COVID-19 infection (OR 1.39, 95% CI 1.03–1.89, $P = 0.033$). The conclusion suggest that male patients and those with severe COVID-19 manifestations demonstrate increased susceptibility to Azvudine-associated injury.

The analysis identified several significant predictors of Azvudine associated hepatotoxicity. Notably, abnormal liver function tests at admission demonstrated the strongest association (OR 5.89, 95% CI 4.28–8.27, $P < 0.001$). Interestingly, the presence of comorbidities did not significantly affect liver function disorders in hospitalized patients. However, the concurrent use of antihypertensive (OR 1.37, 95% CI 1.01–1.88, $P = 0.047$) and anti-thrombotic drugs (OR 2.01, 95% CI 1.48–2.74, $P < 0.001$) was significantly correlated with abnormal liver function. Comparative analysis of baseline laboratory parameters (Figure 2) revealed significant differences ($P < 0.05$) in multiple hepatic markers (ALT, AST, ALP, GGT, TB, and DB), and hematological indices (WBC, RBC, HGB, and PLT) between groups. Notably, coagulation such as the international

TABLE 1 Demographic characteristics of hospitalized COVID-19 patients in Azvudine-associated hepatotoxicity study.

Characteristics		Liver tests			Univariate analysis		Multivariate analysis	
		Total (n = 669, 100%)	Normal (n = 354, 52.9%)	Abnormal (n = 315, 47.1%)	OR (95% CI)	P value	OR (95% CI)	P value
Gender, n (%)	Male	446 (66.7)	218 (61.6)	228 (72.4)	1.63 (1.18–2.27)	0.003**	1.38 (0.94–2.02)	0.102
	Female	223 (33.3)	136 (38.4)	87 (27.6)				
Age, year, median (range)		70.0 (59.0–79.0)	69.5 (59.0–79.0)	70.0 (60.0–79.0)	1.00 (0.99–1.01)	0.589		
Smoke, n (%)	No	581 (86.8)	315 (89.0)	266 (84.4)				
	Yes	88 (13.2)	39 (11.0)	49 (15.6)	1.49 (0.95–2.35)	0.084	1.10 (0.58–2.10)	0.764
Drink, n (%)	No	613 (91.6)	329 (92.9)	284 (90.2)				
	Yes	56 (8.4)	25 (7.1)	31 (9.8)	1.44 (0.83–2.51)	0.197	1.20 (0.56–2.62)	0.640
Allergic, n (%)	No	623 (93.1)	327 (92.4)	296 (94.0)				
	Yes	46 (6.9)	27 (7.6)	19 (6.0)	0.78 (0.42–1.42)	0.417		
Severity of COVID-19, n (%)	Non-severe	352 (52.6)	200 (56.5)	152 (48.3)				
	Severe	317 (47.4)	154 (43.5)	163 (51.8)	1.39 (1.03–1.89)	0.033*	0.90 (0.63–1.28)	0.559
Infection indicators tests on admission	Normal	183 (27.4)	107 (30.8)	76 (24.1)				
	Abnormal	486 (72.6)	247 (69.2)	239 (75.9)	1.36 (0.97–1.92)	0.078	0.91 (0.61–1.37)	0.665
Liver tests on admission	Normal	332 (49.6)	245 (69.2)	87 (27.6)				
	Abnormal	337 (50.4)	109 (30.8)	228 (72.4)	5.89 (4.23–8.27)	<0.001***	5.55 (3.94–7.90)	<0.001***
Comorbidities, n (%)	Liver diseases	88 (13.2)	46 (13.0)	42 (13.3)	1.03 (0.66–1.61)	0.897		
	Renal diseases	208 (30.3)	108 (30.5)	100 (31.8)	1.06 (0.76–1.47)	0.730		
	Hypertension	248 (37.1)	135 (38.1)	113 (35.9)	0.91 (0.66–1.24)	0.545		
	Hyperlipidemia	18 (2.7)	8 (2.3)	10 (3.2)	1.42 (0.55–3.76)	0.468		
	Hypoproteinemia	400 (59.8)	209 (59.0)	191 (60.6)	1.07 (0.78–1.46)	0.674		
	Diabetes	123 (18.4)	68 (19.2)	55 (17.5)	0.89 (0.60–1.32)	0.560		
	Cancer	92 (13.8)	56 (15.8)	36 (11.4)	0.69 (0.43–1.07)	0.101	0.67 (0.38–1.15)	0.145
Azvudine therapy	Treatment duration, days, mean (±SD)	8.8 (±3.8)	8.9 (±3.6)	8.6 (±4.1)	0.98 (0.94–1.02)	0.286		
	Accumulated dose, mg, mean (±SD)	43.1 (±18.8)	44.0 (±17.2)	42.1 (±20.4)	1.00 (0.99–1.00)	0.200		

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TABLE 1 (Continued) Demographic characteristics of hospitalized COVID-19 patients in Azvudine-associated hepatotoxicity study.

Characteristics		Liver tests			Univariate analysis		Multivariate analysis	
		Total (n = 669, 100%)	Normal (n = 354, 52.9%)	Abnormal (n = 315, 47.1%)	OR (95% CI)	P value	OR (95% CI)	P value
Concomitant medication, n (%)	Anti-viral drugs	73 (10.9)	31 (8.8)	42 (13.3)	1.60 (0.98–2.64)	0.060	1.35 (0.77–2.39)	0.303
	Anti-bacterial drugs	632 (94.5)	330 (93.2)	302 (95.9)	1.69 (0.86–3.47)	0.138	1.23 (0.56–2.77)	0.608
	Anti-fungal drugs	69 (10.3)	36 (10.2)	33 (10.5)	1.03 (0.63–1.70)	0.896		
	Respiratory drugs	485 (72.5)	258 (72.9)	227 (72.1)	0.96 (0.68–1.35)	0.813		
	Anti-hypertensive drugs	258 (38.6)	124 (35.0)	134 (42.5)	1.37 (1.01–1.88)	0.047*	1.16 (0.80–1.67)	0.426
	Stain	95 (14.2)	43 (12.1)	52 (16.5)	1.43 (0.93–2.22)	0.108	1.07 (0.64–1.80)	0.792
	Antiplatelet agents	80 (12.0)	42 (11.9)	38 (12.1)	1.02 (0.64–1.63)	0.937		
	Anticoagulant	338 (50.5)	150 (42.4)	188 (59.7)	2.01 (1.48–2.74)	<0.001***	1.79 (1.27–2.54)	0.001**
	Anti-diabetic drugs	137 (20.5)	68 (19.2)	69 (21.9)	1.18 (0.81–1.72)	0.389		
	Glucocorticoids	554 (82.8)	285 (80.5)	269 (85.4)	1.42 (0.95–2.14)	0.095	1.25 (0.79–2.00)	0.349
	NSAIDs	148 (22.1)	75 (21.2)	73 (23.2)	1.12 (0.78–1.62)	0.536		
	PPIs	493 (73.7)	258 (72.9)	235 (74.6)	1.09 (0.78–1.55)	0.614		
	Anti-neoplastic drugs	27 (4.0)	18 (5.1)	9 (2.9)	0.55 (0.23–1.21)	0.149	0.90 (0.34–2.26)	0.821

Abbreviations: COVID-19, Coronavirus disease 2019; OR, Odds ratio; CI, Confidence interval; SD, Standard deviation; NSAIDs, Nonsteroidal anti-inflammatory drugs; PPIs, Proton pump inhibitors.
Note: *means $P < 0.05$. **means $P < 0.01$. ***means $P < 0.001$.

TABLE 2 Demographic characteristics of hospitalized COVID-19 patients in Azvudine-induced hepatotoxicity study.

Characteristics		Liver tests			Univariate analysis		Multivariate analysis	
		Total (n = 294, 100%)	Normal (n = 214, 72.8%)	Abnormal (n = 80, 27.4%)	OR (95% CI)	P value	OR (95% CI)	P value
Gender, n (%)	Male	178 (60.5)	123 (57.5)	55 (68.8)	1.63 (0.95–2.84)	0.080	1.77 (0.994–3.25)	0.057
	Female	116 (39.5)	91 (42.5)	25 (31.3)				
Age, year, median (range)		69.0 (59.0–78.0)	68.0 (58.0–78.0)	70.0 (58.0–78.0)	1.00 (0.98–1.02)	0.933		
Smoke, n (%)	no	266 (90.5)	195 (91.1)	71 (88.8)				
	yes	28 (9.5)	19 (8.9)	9 (11.3)	1.30 (0.54–2.94)	0.538		
Drink, n (%)	no	276 (93.9)	203 (94.9)	73 (91.3)				
	yes	18 (6.1)	11 (5.1)	7 (8.8)	1.77 (0.63–4.67)	0.256		
Allergic, n (%)	no	272 (92.5)	197 (92.1)	75 (93.8)				
	yes	22 (7.5)	17 (7.9)	5 (6.3)	0.77 (0.25–2.03)	0.624		
Severity of COVID-19, n (%)	Non-severe	178 (60.5)	136 (63.6)	42 (52.5)				
	Severe	116 (39.5)	78 (36.5)	38 (47.5)	1.58 (0.94–2.66)	0.086	1.28 (0.72–2.25)	0.399
Infection indicators tests on admission	Normal	99 (33.7)	76 (35.5)	23 (28.8)				
	Abnormal	195 (66.3)	138 (64.5)	57 (71.3)	1.36 (0.79–2.42)	0.276		
Comorbidities, n (%)	Renal diseases	90 (30.6)	63 (29.4)	27 (33.8)	1.22 (0.70–2.10)	0.476		
	Hypertension	110 (37.4)	78 (36.5)	32 (40.0)	1.16 (0.68–1.96)	0.576		
	Hyperlipidemia	7 (2.4)	4 (1.9)	3 (3.8)	2.05 (0.40–9.48)	0.356		
	Hypoproteinemia	168 (57.1)	119 (55.6)	49 (61.3)	1.26 (0.75–2.15)	0.385		
	Diabetes	67 (22.8)	53 (24.8)	14 (17.5)	0.64 (0.33–1.21)	0.188	0.62 (0.29–1.24)	0.191
	Cancer	41 (14.0)	31 (15.0)	10 (12.5)	0.84 (0.38–1.76)	0.662		
Azvudine therapy	Treatment duration, days, mean (±SD)	8.9 (±3.5)	8.7 (±3.5)	9.5 (±3.4)	1.05 (0.98–1.13)	0.163	1.01 (0.93–1.09)	0.903
	Accumulated dose, mg, mean (±SD)	43.5 (±16.5)	42.7 (±16.1)	46.2 (±17.2)	1.01 (1.00–1.03)	0.209		
Concomitant medication, n (%)	Anti-viral drugs	22 (7.5)	10 (4.7)	12 (15.0)	3.60 (1.49–8.89)	0.004**	3.80 (1.47–10.1)	0.006**
	Anti-bacterial drugs	271 (92.5)	196 (91.6)	75 (93.8)	1.38 (0.53–4.29)	0.541		
	Anti-fungal drugs	31 (10.5)	19 (8.9)	12 (15.0)	1.81 (0.82–3.89)	0.132		

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TABLE 2 (Continued) Demographic characteristics of hospitalized COVID-19 patients in Azvudine-induced hepatotoxicity study.

Characteristics		Liver tests			Univariate analysis		Multivariate analysis	
		Total (n = 294, 100%)	Normal (n = 214, 72.8%)	Abnormal (n = 80, 27.4%)	OR (95% CI)	P value	OR (95% CI)	P value
	Respiratory drugs	211 (71.8)	153 (71.5)	58 (72.5)	1.05 (0.60–1.89)	0.865		
	Anti-hypertensive drugs	100 (34.0)	67 (31.3)	33 (41.3)	1.54 (0.90–2.62)	0.111	1.37 (0.75–2.50)	0.305
	Statin	35 (11.9)	22 (10.3)	13 (16.3)	1.69 (0.79–3.51)	0.163	1.51 (0.66–3.41)	0.320
	Antiplatelet agents	32 (10.9)	23 (10.7)	9 (11.3)	1.05 (0.44–2.31)	0.902		
	Anticoagulant	132 (44.9)	79 (36.9)	53 (66.3)	3.35 (1.97–5.82)	<0.001***	3.12 (1.77–5.61)	<0.001***
	Anti-diabetic drugs	58 (19.7)	43 (20.1)	15 (18.8)	0.92 (0.47–1.73)	0.797		
	Glucocorticoids	236 (80.3)	169 (79.0)	67 (83.8)	1.37 (0.71–2.80)	0.361		
	NSAIDs	61 (19.6)	43 (20.1)	18 (22.5)	1.15 (0.61–2.13)	0.651		
	PPIs	223 (75.9)	161 (75.2)	62 (77.5)	1.13 (0.63–2.13)	0.686		
	Anti-neoplastic drugs	14 (4.8)	12 (5.6)	2 (2.5)	0.43 (0.07–1.63)	0.278		

Abbreviations: COVID-19, Coronavirus disease 2019; OR, Odds ratio; CI, Confidence interval; SD, Standard deviation; NSAIDs, Nonsteroidal anti-inflammatory drugs; PPIs, Proton pump inhibitors.
Note: *means $P < 0.05$. **means $P < 0.01$. ***means $P < 0.001$.

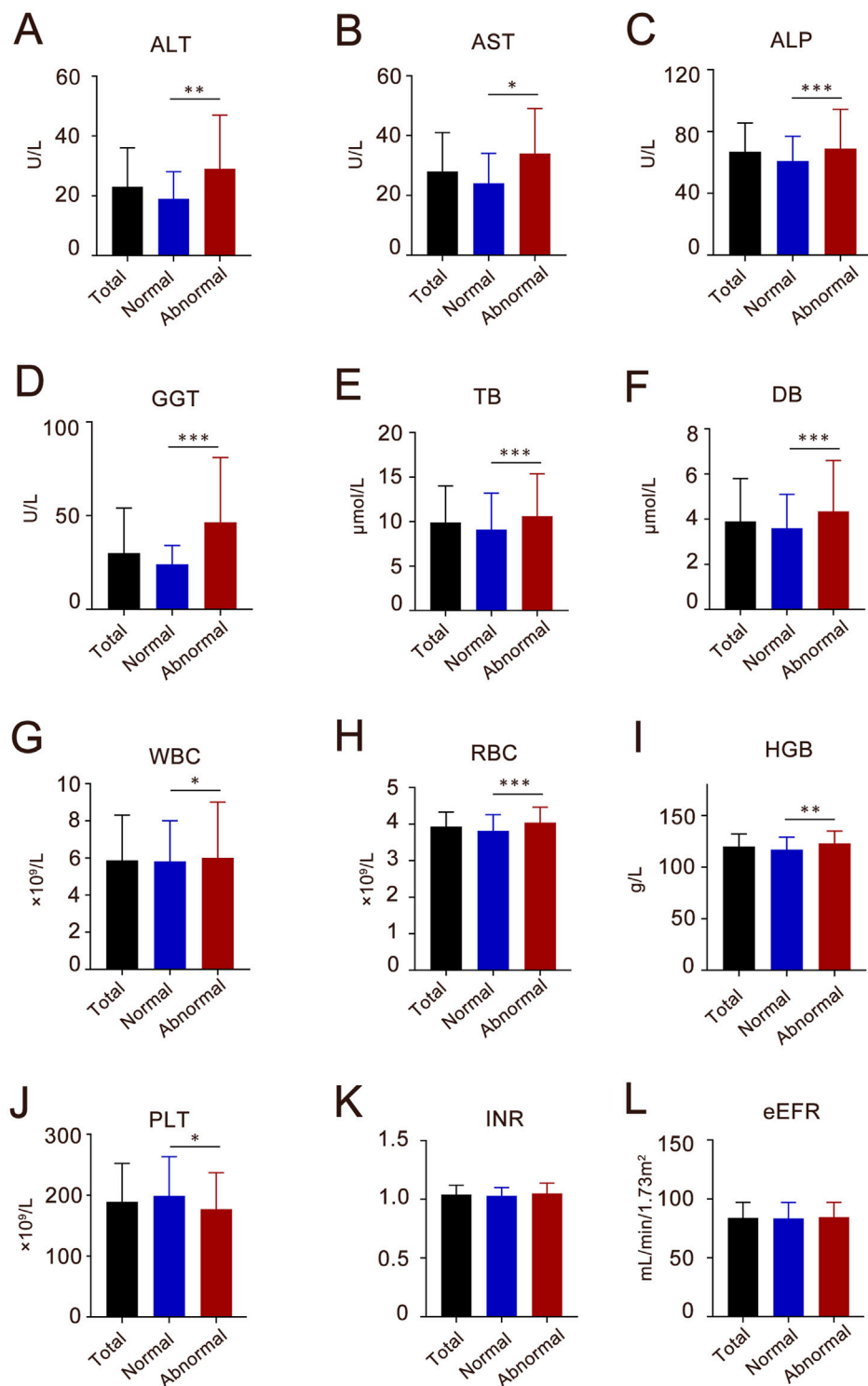


FIGURE 2 The baseline characteristics of liver enzymes, blood components, coagulate and kidney function index of the participants in Azvudine-associated hepatotoxicity study. (A) ALT, alanine aminotransferase; (B) AST, aspartate transaminase; (C) ALP, alkaline phosphatase; (D) GGT, gamma-glutamyltransferase; (E) TB, total bilirubin; (F) DB, direct bilirubin; (G) WBC, white blood cell; (H) RBC, red blood cell; (I) HGB, hemoglobin; (J) PLT, platelet count; (K) INR, international normalized ratio; (L) eGFR, estimated glomerular filtration rate. Each column shows the median (interquartile range, IQR). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

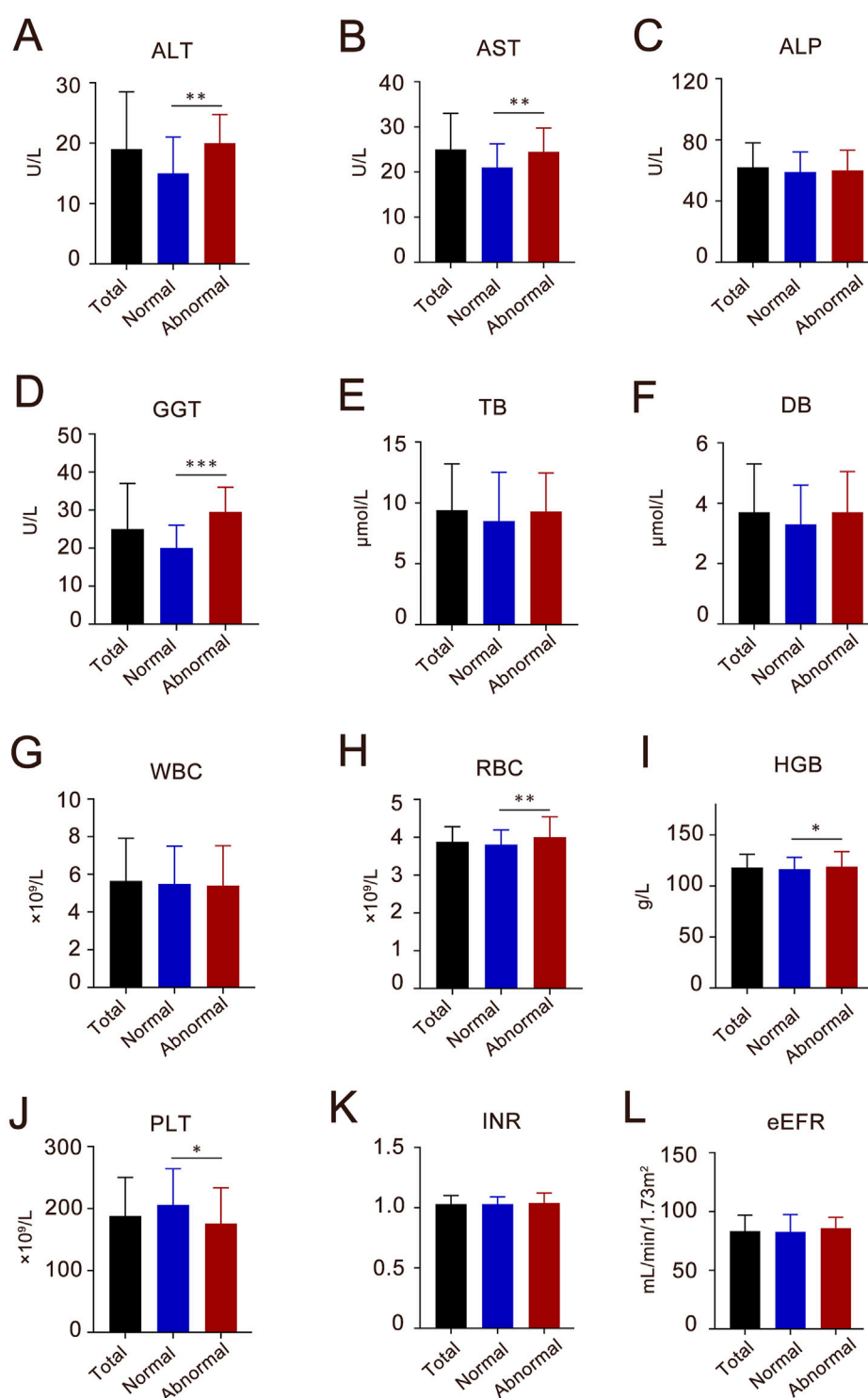


FIGURE 3

The baseline characteristics of liver enzymes, blood components, coagulative and kidney function index of the participants in Azvudine-induced hepatotoxicity study. (A) ALT, alanine aminotransferase; (B) AST, aspartate transaminase; (C) ALP, alkaline phosphatase; (D) GGT, gamma-glutamyltransferase; (E) TB, total bilirubin; (F) DB, direct bilirubin; (G) WBC, white blood cell; (H) RBC, red blood cell; (I) HGB, hemoglobin; (J) PLT, platelet count; (K) INR, international normalized ratio; (L) eGFR, estimated glomerular filtration rate. Each column shows the median (interquartile range, IQR). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

normalized ratio (INR) and renal function parameters such as estimated glomerular filtration rate (eGFR) showed no significant difference intergroup variation.

The multivariate logistic regression analysis incorporating demographic characteristics (gender, smoking, drinking), clinical factors (severity of COVID-19), admission laboratory tests

TABLE 3 The liver tests result of Azvudine on and after admission among hospitalized COVID-19 patients with Azvudine-induced hepatotoxicity.

Liver tests		Azvudine treatment		P value
		On admission	After admission	
ALT (U/L), median (range)	Total	23.0 (14.0–36.0)	26.00 (15.00–49.00)	<0.001***
	Normal	19.0 (12.0–28.0)	18.00 (12.00–25.3)	0.182
	Abnormal	29.0 (19.0–47.0)	52.0 (30.0–79.0)	<0.001***
AST (U/L), median (range)	Total	28.0 (21.0–41.0)	22.00 (16.00–36.00)	0.038*
	Normal	24.0 (18.0–34.0)	17.5 (14.00–22.00)	<0.001***
	Abnormal	34.0 (24.0–49.0)	37.0 (24.0–58.0)	<0.001***
ALP(U/L), median (range)	Total	67.0 (54.5–85.5)	66.00 (54.00–85.00)	0.160
	Normal	61.0 (53.0–77.0)	59.00 (50.00–71.5)	0.918
	Abnormal	69.0 (57.0–94.5)	74.0 (59.0–100.0)	0.025*
GGT (U/L), median (range)	Total	30.0 (20.0–54.0)	34.00 (22.00–63.25)	<0.001***
	Normal	24.0 (17.0–34.0)	23.00 (18.00–31.00)	0.080
	Abnormal	46.5 (27.8–81.0)	74.0 (59.0–100.0)	<0.001***
TB (μmol/L), median (range)	Total	9.9 (7.1–14.0)	9.70 (7.40–13.20)	0.633
	Normal	9.1 (6.9–13.2)	8.6 (6.9–12.0)	0.344
	Abnormal	10.6 (7.7–15.4)	10.7 (7.8–14.8)	0.594
DB (μmol/L), median (range)	Total	3.9 (2.8–5.8)	3.60 (2.60–5.20)	0.410
	Normal	3.6 (2.5–5.1)	3.2 (2.3–4.4)	0.384
	Abnormal	4.4 (3.1–6.6)	4.1 (2.9–6.3)	0.826

Abbreviations: COVID-19, Coronavirus disease 2019; ALT, alanine aminotransferase; AST, aspartate transaminase; ALP, alkaline phosphatase; GGT, gamma-glutamyltransferase; TB, total bilirubin; DB, direct bilirubin.

Note: *means $P < 0.05$. **means $P < 0.01$. ***means $P < 0.001$.

(infection indicator and liver function), and medication history (antiviral drugs, antibacterial drugs, antihypertensive drugs, statins, anticoagulants, glucocorticoids, and antineoplastic drugs) on admission were subsequently incorporated to further identified their associations. (1) Abnormal liver function tests on admission (OR 5.55, 95% CI 3.94–7.90, $P < 0.001$). (2) Antithrombotic medication management (OR 1.79, 95% CI 1.27–2.54, $P = 0.001$). The model demonstrated good discriminatory ability with an area under the ROC curve of 0.756, 95% CI 0.719–0.792, $P < 0.001$, indicating moderate predictive accuracy for liver injury risk in COVID-19 patients receiving Azvudine.

3.3 Characteristics of COVID-19 patients with Azvudine-induced hepatotoxicity

Overall, 80 (27.2%) patients developed hepatotoxicity after Azvudine treatment, while 214 (72.8%) maintained normal. Multivariate analysis identified two independent predictors: concomitant antiviral therapy (adjusted OR 3.60, 95%CI 1.49–8.89, $P = 0.004$); Antithrombotic medication management (adjusted OR 3.35, 95% CI 1.77–5.61, $P < 0.010$). Additionally, the regression model demonstrated acceptable discrimination (AUC 0.712, 95% CI 0.648–0.775, $P < 0.001$). For laboratory examination,

significant post-treatment elevations in hepatic enzymes (baseline levels of ALT, AST, GGT, WBC, HGB, and PLT) were significantly associated with liver injury (all $P < 0.05$, Figure 3). Gender, COVID-19 severity, diabetes, Azvudine therapy, antiviral drugs, and antithrombotic drugs were then incorporated into a multivariate LR model. It was determined that anti-viral (OR 3.80, 95% CI 1.47–10.1, $P = 0.006$) and Anti-thrombotic drugs (OR 3.12, 95%CI 1.77–5.61, $P < 0.001$) were both independent predictors of Azvudine hepatotoxicity. The sensitivity analysis results were consistent with the primary analysis, thus lending support of the accuracy of the conclusion (Supplementary Table S1). Additionally, a significant elevation in liver function (ALT, AST, and GGT) was revealed following Azvudine administration (Table 3), indicating the occurrence of liver injury ($P < 0.05$).

3.4 Concomitant medication of Azvudine-induced hepatotoxicity in COVID-19 patients

To identify concomitant medications significantly associated with Azvudine-induced hepatotoxicity, we conducted focused analysis of pharmacological agents that demonstrated statistical significance ($P < 0.05$) in our multivariate regression model, as

TABLE 4 Concomitant medication of hospitalized COVID-19 patients in Azvudine-induced hepatotoxicity study.

Concomitant medication		Liver tests		Univariate analysis		Multivariate analysis	
		Total (n = 294, 100%)	Normal (n = 214, 72.8%)	Abnormal (n = 80, 27.4%)	OR (95% CI)	P value	P value
Anti-viral drugs	Ganciclovir	17 (5.8)	7 (3.3)	10 (12.5)	4.22 (1.56–12.0)	0.005**	0.008**
		5 (1.7)	3 (1.4)	2 (2.5)	1.80 (0.234–11.1)	0.523	
Anticoagulants	Low Molecular Weight Heparin Calcium	79 (26.9)	44 (20.6)	35 (43.8)	3.01 (1.73–5.23)	<0.001***	<0.001***
	Low Molecular Weight Heparin Sodium	8 (2.7)	4 (1.9)	4 (5.0)	2.76 (0.64–11.9)	0.158	0.146
	Enoxaparin	20 (6.8)	11 (5.1)	9 (11.3)	2.34 (0.91–5.88)	0.071	0.047*
	Nadroparin	23 (7.8)	17 (7.9)	6 (7.5)	0.94 (0.33–2.36)	0.900	
	NOACs	15 (5.1)	12 (5.6)	3 (3.8)	0.66 (0.15–2.13)	0.522	
	Sulotixide	27 (9.2)	23 (10.7)	4 (5.0)	0.44 (0.13–1.18)	0.138	0.269

Abbreviations: COVID-19, Coronavirus disease 2019; OR, Odds ratio; CI, Confidence interval; NOACs, new oral anticoagulants.
Note: *means $P < 0.05$. **means $P < 0.01$. ***means $P < 0.001$.

shown in Table 4. Significant Hepatotoxicity Associations: Ganciclovir (adjusted OR 4.22, 95% CI 1.56–12.0, $P = 0.005$). LMWH showed significant risk (OR 3.01, 95% CI 1.73–5.23, $P < 0.001$). Multivariate Analysis Results: The final predictive model identified three independent pharmacological risk factors: Ganciclovir (adjusted OR 4.11, 95% CI 1.45–12.2, $P = 0.008$). LMWH calcium (adjusted OR 3.00, 95% CI 1.69–5.33, $P < 0.001$). Enoxaparin (adjusted OR 2.68, 95% CI 0.99–7.10, $P = 0.047$). Model Performance: The model achieved acceptable discrimination (AUC 0.674, 95% CI 0.602–0.746, $P < 0.001$). Validation: Robustness confirmed through consistent sensitivity analyses (Supplementary Table S2). All significant associated maintained statistical significance in validation testing.

3.5 Clinical outcome of Azvudine-induced hepatotoxicity, severity, and treatment

The characteristics and outcomes of the Azvudine-induced hepatotoxicity was shown in Table 5. Severity Distribution: Grade 1 (Mild): 64 cases (80.0%); Grade 2 (Moderate): 14 cases (17.5%); Grade 3 (Grade 3+): 2 cases (2.5%). The onset of liver injury occurred at approximately 7 days (range 5–10 days) and showed no significant differences across severity grades.

Causality and Phenotypic Characterization of Azvudine-Induced Hepatotoxicity. RUCAM-based Causality Assessment: Possible association (score 3–5): 41 cases (51.3%); Probable association (score 6–8): 37 cases (46.2%). Highly probable association (score>8): 2 cases (2.5%). Injury Patterns at Presentation: Hepatocellular ($R \geq 5$): 16 cases (20.0%); Cholestatic ($R \leq 2$): 18 cases (22.5%). Mixed ($2 < R < 5$): 46 cases (57.5%).

There was a significant difference between the level of liver injury: mild cases predominantly showed mixed pattern, while severe cases demonstrated hepatocellular predominance. Notably, two cases in the hepatocellular pattern reached grade 3 and one case achieved grade 4, with an increasing AST value of 585 U/L (>10X the upper limit of normal, ULN).

As for treatment patterns: 32/80 (40.0%) patients received hepatoprotective therapy. Medication utilization:Glutathione (19, 23.75%) was the most frequently administered treatment for DILI, followed by Glycyrrhizin (15, 18.75%) and Polyene phosphatidylcholine (6, 7.5%). Treatment Outcome: The majority of patients achieved recovery or improvement after therapy, although two patients showed disease progression despite hepatoprotection. Case presentations of progressive liver injury: One patient initially presented with mild liver injury (Grade 1) with cholestatic pattern that progressed to acute liver failure within 1 week, exhibiting terminal hepatocellular pattern changes with AST levels of 684 U/L, ALT >6000 U/L, ALP 161 U/L, and GGT 79 U/L, whereas TB and DB levels remained normal at 8.3 $\mu\text{mol/L}$ and 5.3 $\mu\text{mol/L}$, respectively. Another patient persistent AST level of >200 U/L with severe liver injury maintained and fatal outcome due to severe pneumonia complications 4 days post discontinuation of Azvudine. Clinical Observation: Although progress cases received hepatoprotective therapy, rapid biochemical deterioration occurred despite standard interventions. Non-hepatic comorbidities contributed to mortality in severe cases.

TABLE 5 Clinical characteristics and outcome of hospitalized COVID-19 patients in Azvudine-induced hepatotoxicity study.

Characteristics		Liver tests				
		Total (n = 80, 100%)	Grade 1 (n = 64, 80.0%)	Grade 2 (n = 14, 17.5%)	Grade 3+ (n = 2, 2.5%)	P value
Time of onset, median (range)		7.0 (5.0–10.0)	6.0 (5.0–10.0)	9.0 (6.8–11.3)	7.5 (5.0–10.0)	0.2204
RUCAM score	3–5	41 (51.3)	34 (53.1)	6 (42.9)	1 (50.0)	0.880
	6–8	37 (46.3)	28 (43.8)	8 (57.1)	1 (50.0)	
	>8	2 (2.5)	2 (3.1)	0(0.0)	0 (0.0)	
Clinical classification	Hepatocyte type	16 (20.0)	8 (12.5)	6 (42.9)	2 (100.0)	0.005**
	Cholestasis type	18 (22.5)	15 (23.4)	3 (21.4)	0 (0.0)	
	Mixed type	46 (57.5)	41 (64.1)	5 (35.7)	0 (0.0)	
Hepatoprotective drugs	Glycyrrhizin drugs	15 (18.8)	12 (18.8)	1 (7.1)	2 (100.0)	0.703
	Glutathione	19 (23.8)	15 (23.4)	3 (24.4)	1 (50.0)	
	Polyene phosphatidyl choline	6 (7.5)	4 (6.3)	2 (14.3)	0 (0.0)	
	Other drugs	6 (7.5)	4 (6.3)	1 (7.2)	1 (50.0)	
Clinical outcome	Recovery	33 (41.3)	29 (45.3)	4 (28.6)	0 (0.0)	0.002**
	Improvement	37 (46.3)	28 (43.8)	9 (64.3)	1 (50.0)	
	Progression	3 (3.8)	1 (1.6)	0 (0.0)	1 (50.0)	
	Unknown	7 (8.8)	6 (9.4)	1 (7.2)	0 (0.0)	
All-cause mortality		5 (7.5)	2 (3.1)	1 (7.2)	2 (100.0)	<0.001***

Abbreviations: COVID-19, Coronavirus disease 2019; RUCAM, Roussel Uclaf Causality Assessment Method.
Note: **means $P < 0.01$. ***means $P < 0.001$.

Mortality analysis in the study cohort. Overall Mortality in entire cohort, 14 (4.76%, 14/294) died after Azvudine therapy. In the normal liver function group, the all-cause mortality rate was 4.21% (9/214), with five patients dying from severe pneumonia, one from MODS, and one from acute heart failure (AHF). The all-cause mortality by hepatotoxicity severity in the liver injury group was 6.25% (5/80), with 2 cases (3.13%, 2/64) Grade 1, 1 case (7.14%, 1/14) Grade 2, and 2 cases (100%, 2/2) Grade 3 or higher. Among which, four patients died from severe pneumonia and one died from MODS. Although there was no significant difference between the normal and abnormal liver function groups, severe liver injury significantly contributed to the progression of the main disease. Liver injury severity correlated with worse clinical outcomes, particularly in patients with pre-existing severe COVID-19. Hepatotoxicity may potentiate disease progression in critical ill patients.

4 Discussion

During the COVID-19 pandemic, the urgent need for the effective antiviral medication has highlighted the importance of rigorous drug safety evaluation. Azvudine has emerged as a promising newly marketed medication, its safety profile, particularly regarding hepatotoxicity remains incompletely characterized, especially for off-label use in hospitalized patients with prolonged symptomatic combined with complex commodities.

In the initial studies, the medication did not demonstrate any significant adverse effects, indicating a seemingly safe profile for its application. Nevertheless, this is the first large-scale evaluation of Azvudine hepatotoxicity in real-world hospitalized patients. The research focus on high-risk population with symptoms prolonged more than 5 days with multiple comorbidities/concomitant medications. Sample size ($n = 1405$) substantially exceeds previous clinical trial cohorts.

Previous studies have documented that hepatocellular injury in 14%–53% of hospitalized COVID-19 patients, typically characterized by an elevation in aminotransferase levels below 5-fold ULN in initial stage of pandemic (Gupta et al., 2020). In our prior single center data demonstrated progress from 51.2% admission prevalence to 70.0% during hospitalization (Chen et al., 2023). Recently, multicenter study enrolling 1246 hospitalized adult patients identified that approximately 58.7% patients had presented with abnormal liver biochemistry and 47.7% had persistent abnormalities up to 6 months post-infection (Rajaram et al., 2024). In our study, the overall hepatotoxicity prevalence: 47.1% consistent with history ranges. Characteristic pattern: isolated with elevated levels of ALT, AST, and GGT, indicating that Azvudine did not increase the risk of hepatotoxicity compared with other anti-viral therapies in COVID-19 treatment.

In our cohort, the incidence of Azvudine-induced hepatotoxicity was 27.2%. Most of cases were characterized by mild-to-moderate

hepatic impairment, corresponding to Grades 1 and 2. In previous studies, varying incidences of hepatic impairment have been documented among COVID-19 patients of diverse types when administered Azvudine therapy, with reported rates of liver injury ranging from 10.5% (Lan et al., 2024) to 17.8% (Yang et al., 2023), 18.6% (Liu S. et al., 2023), 23.0% (Zhi et al., 2023) and 38.4% (He et al., 2023). The retrospective study conducted by Liu et al., among 490 patients with mild COVID-19 who underwent pharmacological treatment, 91 individuals presented with aberrant hepatic function, predominantly manifested as elevated liver enzyme levels (Liu ZQ. et al., 2023). The meticulous study conducted by Li et al., with cohort of 190 patients who received Azvudine demonstrated an incidence of elevated ALT of 38.4% (73/190) (He et al., 2023). The majority of these cases, specifically 93.2% (68/73) patients exhibited an increase within one-fold the ULN and zero case up to 5-fold, which is indicative of a benign pattern typically observed in clinical trials. Age specific hepatotoxicity profile of Azvudine therapy. Elderly patients aged over 75 years old with the rate of abnormal liver function of 23.0% (17/74), among which 6 occurrences of elevated ALT, 4 of elevated AST, and 7 of elevated GGT (Zhi et al., 2023). Notably, the incidence of Azvudine-induced DILI was observed to be relatively as low as 0.68% (2/294) in our study. Consistent findings were reported in another cohort by Zhou et al., with Azvudine-induced DILI of 0.64% (2/311) in the treatment for moderate to severe COVID-19 patients (Zhou et al., 2023). Liu et al. conducted the study involving 294 patients, of which 17 cases of DILI were identified (Liu et al., 2024). The higher incidence may be attributed to the inclusion of patients with pre-existing elevated liver function at the enrollment stage, which could not only increase the risk of occurrence but also confounds non-pharmacological factors. In summary, the aggregate data from these studies suggest that Azvudine was associated with a favorable safety profile in the context of COVID-19 therapy.

Medications have been identified as one of the causes of hepatocellular damage in COVID-19 infections (Zhong et al., 2020; Wen-Shu Hu, 2022; Rodriguez-Espada et al., 2024). In previous researches, antiviral drugs, Remdesivir, and Nirmatrelvir-Ritonavir have been reported to have greater risk of developing drug-induced hepatotoxicity (Allison et al., 2023; Xie et al., 2020; Naserallah et al., 2022). During the Azvudine therapy in our cohort, the value of liver enzymes increased significantly, revealing the potential hepatotoxicity. However, the mechanism underlying Azvudine-induced hepatic injury in COVID-19 patients remained unclear. Naveen et al. have demonstrated that Azvudine could cause mitochondrial ROS induction by two functional groups on sugar, Azido at 4'-poisoning and fluorine at 2'-poisoning, induce ROS generation with a time- and dose-dependent manner, prompt mitochondrial-mediated apoptosis (Kumar et al., 2024b; Kumar et al., 2024a). However, due to its minimal plasma protein binding affinity, Azvudine exhibited a propensity for accumulation in the thymus and was excreted in its parent form via renal clearance, which seems to be a safety pattern in the liver system (He et al., 2023; Liu S. et al., 2023; Sheng et al., 2024). Based on current pharmacokinetic and toxicity data, we assumed that the administration of Azvudine might lead to a dose-dependent intrinsic hepatotoxicity due to the accumulation exceeding the threshold level as a consequence of impaired excretion.

Interestingly, in the present study only concomitant antiviral and anticoagulant drugs were independent risk factors for Azvudine-induced hepatotoxicity, whereas other factors, including gender, age, severity of COVID-19, and comorbidities, were not significantly associated with the risk of liver injury. Further examination revealed that the co-administration of Ganciclovir, Low-Molecular-Weight Heparin Calcium, and Enoxaparin increased the risk. Literatures have indicated an increased likelihood of adverse drug reactions with the concurrent use of multiple antiviral agents (Liu et al., 2024). This might be attributed to competitive targeting by these drugs, resulting in cellular damage (Abdallah et al., 2014). Therefore, the concomitant use of multiple antiviral agents in clinical settings was not advocated. Additionally, it is suggested the concurrent use of anticoagulant drugs contributed not only the Azvudine but also Azvudine-induced hepatotoxicity. These conclusions aligned with previous studies, which identified anticoagulant as an independent risk factor for liver injury and increased 28-day mortality in COVID-19 patients, underscoring the potential correlation that warrants further investigation (Chen et al., 2024; Lu et al., 2024). Retrospective cohort study conducted by Yang et al., anticoagulants (LMWH and Fondaparinux) were associated with liver dysfunction with occurrence rate of 17.1% (79/463) in pulmonary embolism patients, recommending transit to oral anticoagulants if possible (Yang et al., 2020). These findings were also recommended in our research as the new oral anticoagulants (NOACs) showed no significant difference in hepatotoxicity. Elder age and hypoproteinemia have been identified as risk factors for the progress of Azvudine-induced hepatotoxicity in previous studies (Lan et al., 2024; Zhi et al., 2023; Lu et al., 2024). However, the factors were not obvious in our study, possibly due to the inclusion of patients with a median age of >65 years, and the prevalence of critically ill patients contributed to lower baseline albumin level, which could have obscured the observed differences in the analysis.

In this study, the relatively low RUCAM scores, with only two cases indicating a highly probable association, suggested a tenuous causal relationship between Azvudine and hepatotoxicity (Danan and Teschke, 2018; Caines and Moonka, 2024; Zhao et al., 2024). This observation likely stems from multiple factors. Notably, polypharmacy was prevalent, the concurrent use of hepatotoxic agents with established hepatotoxic potential, such as statins and NSAIDs, complicated the assessment of Azvudine's specific contribution to hepatotoxicity (Teschke et al., 2022; Russo et al., 2014; Zoubek et al., 2020). Additionally, the absence of rechallenge data posed a significant challenge. Most patients did not reintroduce the suspected medication post-discontinuation, creating an evidentiary gap in a critical RUCAM evaluation component. This lack of rechallenge documentation made definitively establishing a drug-hepatotoxicity link particularly difficult. Furthermore, the study's relatively small positive sample size may have contributed to the lower RUCAM scores. Future research will address these limitations through large-scale studies and more comprehensive hepatotoxicity assessments, with the goal of clarifying the causal relationship between Azvudine and hepatotoxicity.

Although our study has several limitations, the conclusion provide great significance insights into hepatotoxicity associated with antiviral therapy in COVID-19 patients. First, as retrospective analysis, potential biases may existed due to incomplete or inconsistent data recording in electronic medical records (e.g.,

Incomplete body mass index). Second, the available data on combining medications were insufficient for robust statistical analysis, and the result was still suspicious and required further validation in future studies. Additionally, the lack of standardized follow up protocols limited our ability to monitor liver injury progression and management during Azvudine therapy in real time. To address these limitations, multicenter prospective study with larger sample sizes to comprehensively assess the real-world hepatotoxicity profile of Azvudine. The future research will incorporate structured follow-up schedules, standardized laboratory monitoring, and detailed documentation of concomitant medications to enhance data reliability and clinical applicability.

5 Conclusion

Our investigation demonstrated a significant association between Azvudine and hepatotoxicity, with a notably elevated incidence of 27.2%. Most of these hepatotoxic events were characterized by mild severity and were reversible upon drug withdrawal. Thus, it was essential for clinicians to monitor the hepatic function vigilantly throughout the therapeutic course. Moreover, the incidence of Azvudine-induced hepatotoxicity appeared to be exacerbated by the concomitant administration of antiviral and anticoagulant therapies, specifically Ganciclovir, Low-molecular-weight heparin calcium, and Enoxaparin. Therefore, we do not recommend the concurrent use of Ganciclovir with Azvudine, and instead we suggest considering NOACs as a preferable alternative to LMWH for anticoagulation therapy in patients receiving Azvudine therapy.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by the Ethics Committee of the Renmin Hospital of Wuhan University. The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because This study is a single-center retrospective cohort study.

Author contributions

YX: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation,

Visualization, Writing – original draft, Writing – review and editing. HX: Data curation, Formal Analysis, Writing – original draft. CS: Investigation, Methodology, Software, Writing – original draft. XG: Data curation, Formal Analysis, Writing – review and editing. YC: Data curation, Methodology, Resources, Writing – review and editing. CH: Data curation, Writing – review and editing. FM: Funding acquisition, Supervision, Writing – review and editing. GY: Conceptualization, Supervision, Validation, Visualization, Writing – review and editing. JY: Conceptualization, Supervision, Validation, Visualization, Writing – review and editing.

Funding

The author(s) declare that no financial support was received for the research and/or publication of this article.

Acknowledgments

We are grateful to all the participants in this study. We also extend our sincere thanks to Dr. Zhou Liu and Palpasa Sharestha for their professional language editing, which greatly improved the clarity and quality of this manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2025.1558054/full#supplementary-material>

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OPEN ACCESS

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RECEIVED 16 January 2025

ACCEPTED 16 June 2025

PUBLISHED 26 June 2025

CORRECTED 09 September 2025

CITATION

Che X and Zhu Y (2025) Gastrointestinal stromal tumors with the use of ripretinib and sunitinib: real-world adverse event analysis based on the FDA adverse event reporting system (FAERS). *Front. Pharmacol.* 16:1561937. doi: 10.3389/fphar.2025.1561937

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Gastrointestinal stromal tumors with the use of ripretinib and sunitinib: real-world adverse event analysis based on the FDA adverse event reporting system (FAERS)

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Objective: This study aims to analyze potential adverse events (AEs) associated with ripretinib and sunitinib in gastrointestinal stromal tumor (GIST) treatment using data from the FDA Adverse Event Reporting System (FAERS). The findings provide insights for future research to improve the safety and clinical management of ripretinib and sunitinib.

Methods: Adverse Drug Event (ADE) reports related to ripretinib and sunitinib were extracted from the FAERS database, covering the period from Q2 2020 to Q4 2024 and Q1 2006 to Q4 2024, respectively. ADEs were classified and described according to Preferred Terms (PTs) and System Organ Classes (SOCs) in the Medical Dictionary for Regulatory Activities (MedDRA). Disproportionality analysis, including Reporting Odds Ratio (ROR), Proportional Reporting Ratio (PRR), Bayesian Confidence Propagation Neural Network (BCPNN), and Multi-Item Gamma Poisson Shrinker (MGPS), was employed to identify significant signals.

Results: A total of 3,636 and 34,768 ADE reports related to ripretinib and sunitinib were identified using four disproportionality analysis methods. The top five ADR signals for ripretinib include hepatic embolization, tumor compression, hyperkeratosis, tumor excision and tumor pain. For sunitinib, the five strongest ADR signals are metastatic renal cell carcinoma, diffuse uveal melanocytic proliferation, renal cancer metastasis, connective tissue neoplasm and salivary gland fistula. Both drugs share significant ADRs including palmar-plantar erythrodysesthesia syndrome, disease progression and hyperkeratosis. Furthermore, subgroup analysis was conducted to explore sex difference in ripretinib and sunitinib.

Conclusion: This study validated known AEs and identified new potential safety signals associated with ripretinib and sunitinib in GIST treatment. These findings contribute to the understanding of ripretinib and sunitinib, providing valuable evidence for improving its clinical use.

KEYWORDS

ripretinib, sunitinib, adverse events, FAERS, GIST treatment

1 Introduction

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the gastrointestinal tract, primarily affecting the stomach and small intestine, with an estimated global incidence of 10–15 cases per million individuals (Søreide et al., 2016) (Kelly et al., 2021). Targeted cancer therapies have emerged as a significant advancement in prolonging the survival of patients with advanced GIST (3). Approximately 80% of GIST cases harbor activating mutations in the stem cell factor receptor (KIT) receptor tyrosine kinase gene, while 5%–10% involve mutations in the platelet-derived growth factor receptor alpha (PDGFRA) gene (Dhillon, 2020). Identifying the molecular subtypes of GIST is of critical importance in clinical treatment. Although imatinib is highly effective as first-line therapy for GISTs, secondary resistance mutations frequently arise, necessitating subsequent second-line treatment with sunitinib (Cohen et al., 2021). Sunitinib, an oral multi-targeted tyrosine kinase inhibitor (TKI), mediates antitumor activity by simultaneously inhibiting angiogenesis and tumor cell proliferation through targeting vascular endothelial growth factor receptors (VEGFRs), platelet-derived growth factor receptors (PDGFRs), and KIT (6).

In GIST patients exhibiting progression after sequential treatment with imatinib and sunitinib, tumor cells develop complex kinase conformational alterations via acquired mutations, significantly reducing the efficacy of conventional TKIs. This clinical challenge prompted the development of novel targeted therapies, notably ripretinib, a breakthrough fourth-line agent. Ripretinib, the first FDA-approved broad-spectrum kinase inhibitor for fourth-line GIST treatment (Ripretinib for gastrointestinal stromal tumours, 2021), uniquely targets both the kinase activation loop and switch pocket, effectively suppressing diverse resistance mutations including KIT and PDGFRA variants (Goggin et al., 2022).

In May 2020, ripretinib received FDA approval based on results from the Phase III INVICTUS trial, indicated for advanced GIST patients with disease progression following previous treatments with imatinib, sunitinib, and regorafenib. Its favorable safety profile and enhanced tolerability facilitate prolonged disease control in advanced stages, representing a significant advancement in addressing TKI resistance. Although Ripretinib demonstrates favorable safety and tolerability profiles in clinical trials, adverse events (AEs) remain inevitable in real-world applications. In the phase III INVICTUS trial, the most common AEs (incidence >20%) included alopecia, myalgia, nausea, fatigue, palmar-plantar erythrodysesthesia, and diarrhoea (Blay et al., 2020). Identifying these AEs is crucial for ensuring patient safety and optimizing clinical outcomes, necessitating the application of data mining algorithms to detect potential safety signals of Ripretinib in real-world settings.

The U.S. Food and Drug Administration Adverse Event Reporting System (FAERS) is one of the largest post-marketing safety monitoring databases, and the reliability and validity of its data have been widely recognized in the industry (Chen et al., 2021; Yu et al., 2021). Using the FAERS database, pharmacovigilance research related to ripretinib and sunitinib was conducted to identify adverse events (AEs) not described on the drug label. These findings not only enhance research on ripretinib by comparing classical drug-sunitinib, providing valuable references for clinical drug use.

2 Materials and methods

2.1 Data source

The FAERS database is a publicly accessible, voluntary, and spontaneous reporting system designed for post-marketing surveillance of FDA-approved drugs. It collects adverse drug event (ADE) reports from healthcare professionals, patients, and pharmaceutical manufacturers worldwide, reflecting real-world ADE occurrences (Guo et al., 2022; Pan et al., 2024). This study aims to systematically evaluate the post-marketing safety of ripretinib and sunitinib (Figure 1). Only reports of ripretinib and sunitinib as primary suspect drugs were included. For cases with duplicate CASEIDs, the record with the most recent FDA_DT or the highest PRIMARYID was retained. Subsequently, AEs were standardized, classified, and described based on the Preferred Terms (PT) and System Organ Classes (SOC) defined in the Medical Dictionary for Regulatory Activities (MedDRA 26.1). Some of the analysis results were computed using R software 4.4.3, with packages including “dplyr”, “ggplot2”, “forestplot” and “data.table” for data handling and visualization.

Since the FAERS database is publicly accessible and patient records are anonymized and de-identified, this study does not involve informed consent or ethical approval.

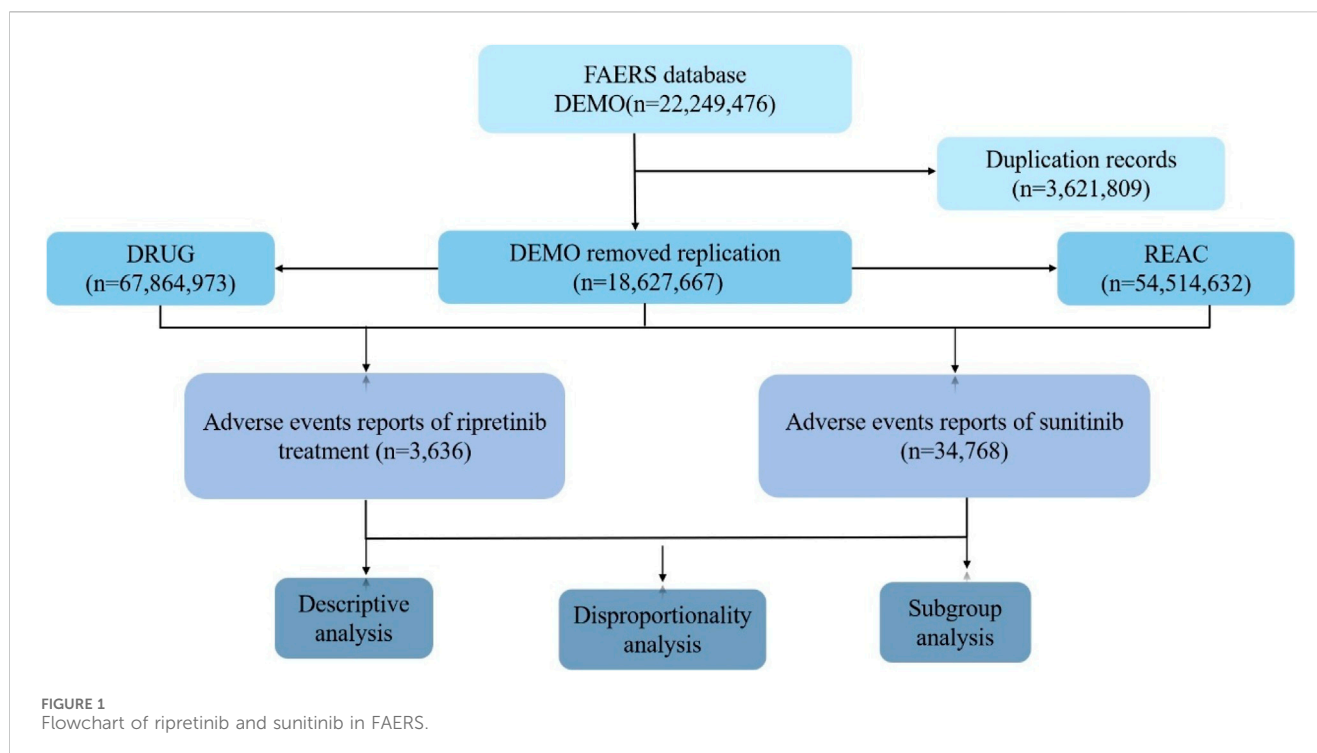
2.2 Methods of data analysis

Disproportionality analysis is a tool used to generate hypotheses about specific drugs and AEs and to clinically evaluate potential case reports (Zh et al., 2025). Four methods were employed for ADE signal mining, including Reporting Odds Ratio (ROR), Proportional Reporting Ratio (PRR), Bayesian Confidence Propagation Neural Network (BCPNN), and Multi-item Gamma Poisson Shrinker (MGPS) (Luo et al., 2025). The ROR and PRR algorithms are non-Bayesian methods, with the advantage of ROR being its ability to correct bias when event reports are limited, while the advantage of PRR is lower sensitivity by the omission of certain AEs (Li et al., 2025). The MHRA method extends PRR by combining PRR values with absolute report numbers and chi-square values, ensuring a minimum number of case combinations (Zhou et al., 2023). The BCPNN method can perform early signal detection even with limited or missing data, and as the number of reports increases, the detection results become more stable (Godfrey et al., 2025). Compared to non-Bayesian algorithms, Bayesian algorithms have higher specificity, signal stability, and lower misclassification probabilities. The data analysis process in this study is illustrated in Figure 1. The formulas and signal detection criteria of the four methods refer to the methods given in previous literature (Xiong et al., 2023; Kuai et al., 2024). In this study, AE signals were considered significant only if they met the criteria for all four algorithms simultaneously.

3 Results

3.1 General analysis of AEs in ripretinib and sunitinib

A total of 3,636 and 34,768 individual AE reports for ripretinib and sunitinib, respectively, were extracted from the FAERS database



(Table 1). In terms of gender distribution, 54.2% of ripretinib-related reports were male, compared to 59.3% in the sunitinib group. Female patients accounted for 43.7% in the ripretinib group and 31.8% in the sunitinib group. Age information was missing in 52.0% of ripretinib cases and 24.8% of sunitinib cases. Among the available data, patients aged ≥ 65 years comprised the largest proportion in both groups (29.5% for ripretinib and 38.1% for sunitinib), followed by those aged 18–64 years (18.4% vs 36.9%). Reports involving patients under 18 years were rare in both groups. Weight data were largely incomplete, with 91.1% missing in the ripretinib group and 73.4% in the sunitinib group. Among the reports with available weight, most patients weighed between 50 and 100 kg in both groups. Regarding reporter type, most ripretinib cases were submitted by consumers (61.4%), whereas sunitinib cases were more frequently reported by physicians (29.9%) and consumers (38.7%). Reports from other healthcare professionals, such as pharmacists and non-physician health professionals, were more prevalent in the sunitinib group. The largest proportion of outcomes reported for ripretinib was categorized as “hospitalization,” accounting for 18.2% of the total cases except missing cases. “other outcomes” and “death” were also important outcomes, comprising 11.4% and 10.6% of total reports. For sunitinib, death was the most frequent outcome (29.8% of cases), followed by “other outcomes” (22.1%) and “hospitalization” (21.3%). Geographically, the majority of ripretinib reports originated from the United States (93.1%), whereas sunitinib reports were more geographically diverse, with 49.1% from the United States, followed by notable contributions from Japan (5.6%), Argentina (5.3%), China (5.2%), and India (4.0%). The annual distribution of reports revealed distinct patterns between sunitinib and ripretinib. Sunitinib-related reports have been submitted consistently since 2006, with a marked increase

beginning in 2010 and peaking around 2016. After 2017, the number of reports gradually declined. In contrast, ripretinib-related reports only appeared from 2020 onwards, aligning with its later market approval. Since then, the number of ripretinib reports has shown a steady increase, surpassing sunitinib in total annual reports by 2022 (Figure 2).

3.2 Signal detection at the SOC level

Ripretinib and sunitinib related AEs were associated with 27 distinct system organ classes (SOCs), of which 7 and 10 met the criteria of all four disproportionality analysis methods (Figure 3). Among all SOC, the most commonly reported in ripretinib were general disorders and administration site conditions ($n = 2579$), skin and subcutaneous tissue disorders ($n = 1637$) and gastrointestinal disorders ($n = 1632$). General disorders and administration site conditions ($n = 27,124$), gastrointestinal disorders ($n = 23,592$) and investigations ($n = 11,178$) were most frequent SOC in sunitinib, which mostly consistent with ripretinib. Moreover, as for ripretinib, attention should also be paid to less common SOC such as musculoskeletal and connective tissue disorders and neoplasms benign, malignant and unspecified (incl cysts and polyps) in clinical practice.

3.3 Signal detection at the PT level

Based on disproportionality analysis, both ripretinib and sunitinib exhibit significant ADR signals in Table 2, 3. The top five ADR signals for ripretinib include hepatic embolization (ROR = 177.62, 95%CI: 55.92–564.17), tumor compression (ROR = 159.5,

TABLE 1 Clinical characteristics of patients with GIST in sunitinib and ripretinib from the FAERS database.

Characteristics	Ripretinib	Sunitinib
Number of reports	3636	34,768
Gender		
Male	1969 (54.2%)	20,608 (59.3%)
Female	1589 (43.7%)	11,072 (31.8%)
Missing	78 (2.1%)	3088 (8.9%)
Age (years), n (%)		
<18	2 (0.1%)	63 (0.2%)
18–64	670 (18.4%)	12,821 (36.9%)
≥65	1074 (29.5%)	13,263 (38.1%)
Missing	1890 (52.0%)	8621 (24.8%)
Weight (kg), n (%)		
<50	39 (1.1%)	559 (1.6%)
50–100	252 (6.9%)	7631 (21.9%)
>100	31 (0.9%)	1054 (3.0%)
Missing	3314 (91.1%)	25,524 (73.4%)
Reporter, n (%)		
Consumer	2232 (61.4%)	13,449 (38.7%)
Health Professional	715 (19.7%)	939 (2.7%)
Physician	598 (16.4%)	10,399 (29.9%)
Other Professional	0	5915 (17.0%)
Pharmacist	86 (2.4%)	2867 (8.2%)
Missing	5 (0.1%)	1199 (3.5%)
Outcome, n (%)		
Death	384 (10.6%)	10,353 (29.8%)
Hospitalization	662 (18.2%)	7422 (21.3%)
Life threatening	11 (0.3%)	767 (2.2%)
Disability	0	145 (0.4%)
Other outcomes	415 (11.4%)	7657 (22.1%)
Missing	2164 (59.5%)	8424 (24.2%)
Country, n (%)		
The United States	3385 (93.1%)	17,065 (49.1%)
France	60 (1.7%)	
Canada	46 (1.3%)	
The United Kingdom	14 (0.4%)	
Australia	13 (0.4%)	
Japan		1947 (5.6%)
Argentina		1837 (5.3%)

(Continued in next column)

TABLE 1 (Continued) Clinical characteristics of patients with GIST in sunitinib and ripretinib from the FAERS database.

Characteristics	Ripretinib	Sunitinib
China		1794 (5.2%)
India		1390 (4.0%)

95%CI: 87.32–291.34), hyperkeratosis (ROR = 90.43, 95%CI: 74.54–109.7), tumor excision (ROR = 82.25, 95%CI: 48.45–139.64), and tumor pain (ROR = 74.04, 95%CI: 48.08–114.03). For sunitinib, the five strongest ADR signals are metastatic renal cell carcinoma (ROR = 270.16, 95%CI: 245.71–297.04), diffuse uveal melanocytic proliferation (ROR = 133.8, 95%CI: 43.15–414.88), renal cancer metastasis (ROR = 127.34, 95%CI: 107.62–150.66), connective tissue neoplasm (ROR = 109.48, 95%CI: 44.39–270.01), and salivary gland fistula (ROR = 109.48, 95%CI: 30.54–392.42).

Both drugs share significant ADRs including palmar-plantar erythrodysesthesia syndrome (Ripretinib: ROR = 27.05; Sunitinib: ROR = 27.73), disease progression (Ripretinib: ROR = 31.03; Sunitinib: ROR = 23.93), and hyperkeratosis (Ripretinib: ROR = 90.43; Sunitinib: ROR = 18.58), indicating possible similarities in pharmacological effects or biological mechanisms.

However, significant differences exist in the ADR profiles between the two drugs. Ripretinib tends to cause tumor-associated direct complications such as tumor compression and surgical-related reactions, while Sunitinib predominantly involves metastasis of specific tumor types and rare pathological conditions like renal cancer metastasis and uveal melanocytic proliferation. Consequently, individualized risk management strategies should be implemented based on their distinct ADR characteristics in clinical practice.

3.4 Gender-based differences in AEs

Subgroup analyses were conducted to identify sex-specific patterns of AEs associated with ripretinib and sunitinib. For ripretinib in Figure 4, male patients most frequently reported alopecia (n = 187), extra dose administered (n = 224), and neoplasm progression (n = 144), with significant disproportionality signals (RORs: 33.84, 44.45, and 32.06, respectively). Notably, tumor compression (ROR: 179.04; 95% CI: 84.76–378.19), tumor excision (ROR: 118.32), and hair texture abnormal (ROR: 107.19) showed the strongest associations. Other relevant AEs included hyperkeratosis (n = 59, ROR: 87.03) and palmar-plantar erythrodysesthesia syndrome (n = 60, ROR: 19.48). Among females, the top reported AEs were underdose (n = 161), extra dose administered (n = 122), and palmar-plantar erythrodysesthesia syndrome (n = 73). Tumor compression (ROR: 139.55; 95% CI: 43.28–449.95), hyperkeratosis (ROR: 112.33), and product coating issues (ROR: 82.42) demonstrated strong disproportionality.

For sunitinib in Figure 5, males most commonly reported renal cell carcinoma (n = 591), metastatic RCC (n = 486), neoplasm progression (n = 1,190), and oral pain (n = 569). The strongest

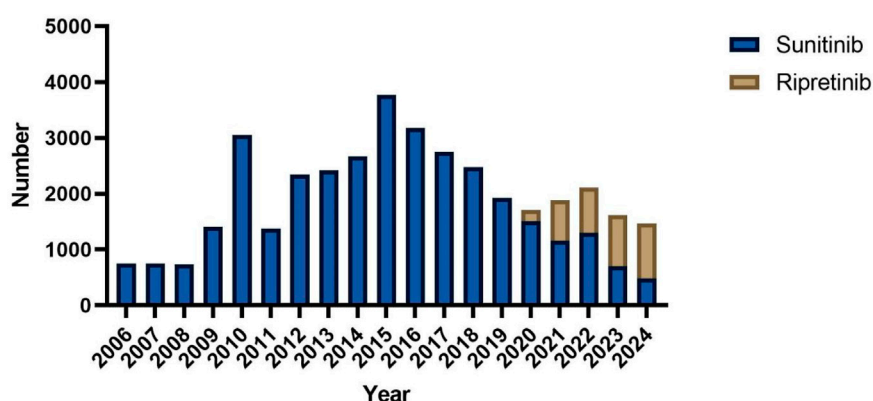


FIGURE 2
Comparison of reporting year between sunitinib and ripretinib.

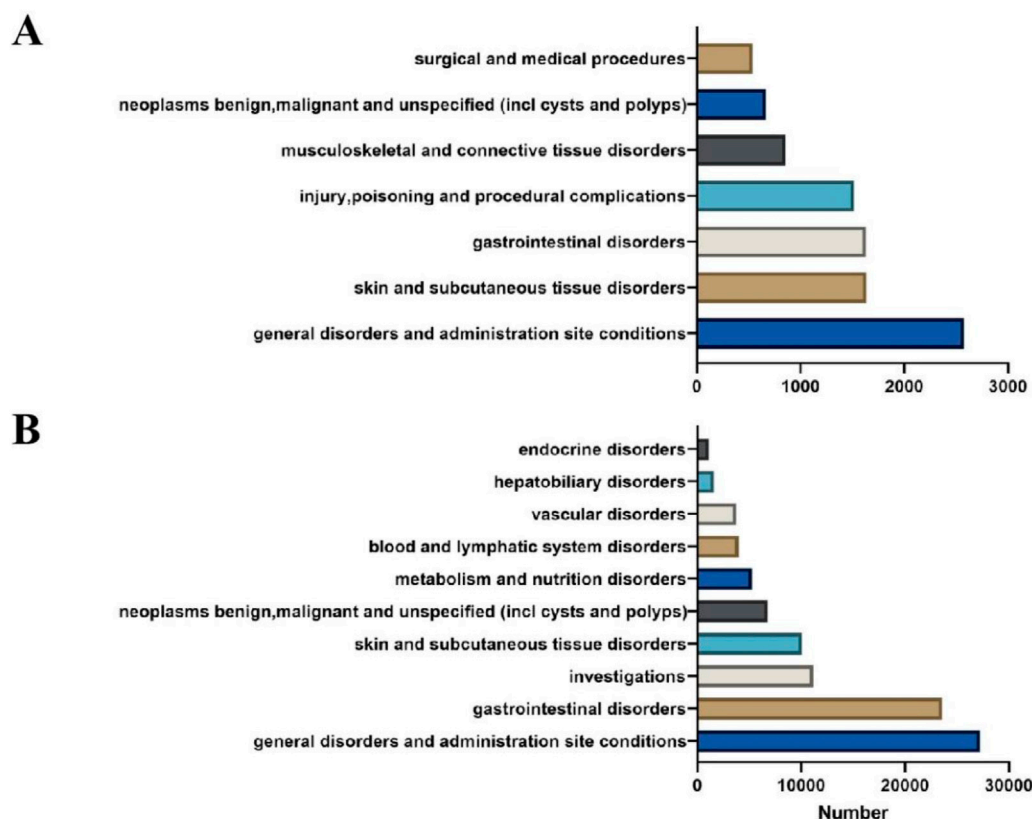


FIGURE 3
Comparison between ripretinib and sunitinib at the SOC level. (A) AE signals at the SOC level in ripretinib. (B) AE signals at the SOC level in sunitinib.

disproportionality was observed for metastatic RCC (ROR: 151.46; 95% CI: 135.0–169.94) and diffuse uveal melanocytic proliferation (BDUMP) (ROR: 128.53; 95% CI: 37.62–439.08), the latter being a rare but fatal ocular event. In female patients, renal cell carcinoma ($n = 245$), metastatic RCC ($n = 202$), and oral pain ($n = 485$) were the most frequent. Disproportionality was greatest for metastatic RCC (ROR: 446.23; 95% CI: 372.83–534.09), renal cancer metastatic (ROR: 211.01), and connective tissue neoplasm (ROR: 160.04).

4 Discussion

The ADR analysis derived from the FAERS database reveal critical similarities and differences between ripretinib and sunitinib, two widely used multi-target TKIs. Expanding upon previous studies that examined ripretinib individually, this analysis includes a comparative approach incorporating subgroup analysis to provide a more comprehensive evaluation of their ADR characteristics.

TABLE 2 Top 30 AE analysis ranked by ROR value in ripretinib

PT	N	ROR (95%CI)	PRR (χ^2)	EBGM(EBGM05)	IC(IC025)
Hepatic embolisation	3	177.62 (55.92–564.17)	177.58 (505.1)	170.32 (64.76)	7.41 (5.93)
Tumour compression	11	159.5 (87.32–291.34)	159.36 (1666.95)	153.5 (92.72)	7.26 (6.41)
Hyperkeratosis	106	90.43 (74.54–109.7)	89.71 (9101.86)	87.83 (74.72)	6.46 (6.17)
Tumour excision	14	82.25 (48.45–139.64)	82.16 (1100.66)	80.59 (51.75)	6.33 (5.58)
Tumour pain	21	74.04 (48.08–114.03)	73.93 (1484.3)	72.65 (50.62)	6.18 (5.56)
Ephelides	8	69.83 (34.71–140.47)	69.78 (533.41)	68.64 (38.25)	6.1 (5.13)
Scan abnormal	4	50.85 (18.97–136.34)	50.84 (193.07)	50.24 (22.01)	5.65 (4.35)
Extra dose administered	347	48.44 (43.51–53.92)	47.18 (15,517.6)	46.66 (42.66)	5.54 (5.39)
Gastric neoplasm	6	43.71 (19.55–97.73)	43.69 (247.68)	43.25 (22.06)	5.43 (4.34)
Product coating issue	9	42.36 (21.96–81.7)	42.33 (359.5)	41.91 (24.19)	5.39 (4.47)
Abdominal cavity drainage	4	41.76 (15.59–111.83)	41.75 (157.5)	41.34 (18.13)	5.37 (4.07)
Neoplasm progression	244	31.03 (27.33–35.24)	30.47 (6909.28)	30.26 (27.21)	4.92 (4.73)
Skin hypertrophy	17	28.23 (17.52–45.5)	28.2 (442.97)	28.01 (18.79)	4.81 (4.13)
Acrochordon	8	28.06 (14–56.25)	28.04 (207.23)	27.86 (15.57)	4.8 (3.83)
Melanocytic naevus	27	27.94 (19.13–40.81)	27.88 (695.22)	27.7 (20.18)	4.79 (4.24)
Palmar-plantar Erythrodysaesthesia syndrome	136	27.05 (22.83–32.04)	26.78 (3354.45)	26.61 (23.09)	4.73 (4.49)
Cancer pain	14	25.96 (15.34–43.91)	25.93 (333.5)	25.78 (16.6)	4.69 (3.94)
Nipple disorder	3	21.18 (6.81–65.87)	21.18 (57.38)	21.07 (8.15)	4.4 (2.95)
Spinal cord neoplasm	4	20.78 (7.78–55.5)	20.77 (74.89)	20.67 (9.08)	4.37 (3.07)
Biopsy	4	20.67 (7.74–55.22)	20.67 (74.48)	20.57 (9.04)	4.36 (3.07)
Abdominal neoplasm	4	20.65 (7.73–55.15)	20.64 (74.38)	20.54 (9.03)	4.36 (3.07)
Nutritional condition abnormal	3	20.22 (6.5–62.86)	20.21 (54.52)	20.12 (7.79)	4.33 (2.88)
Hepatic neoplasm	14	19.67 (11.63–33.26)	19.65 (246.66)	19.56 (12.6)	4.29 (3.54)
Solar lentigo	4	19.25 (7.21–51.43)	19.25 (68.89)	19.16 (8.42)	4.26 (2.97)
Underdose	286	18.7 (16.63–21.03)	18.31 (4666.06)	18.24 (16.53)	4.19 (4.02)
Skin papilloma	16	18.05 (11.04–29.5)	18.03 (256.26)	17.96 (11.9)	4.17 (3.46)
Metastases to peritoneum	9	17.49 (9.08–33.66)	17.47 (139.2)	17.41 (10.06)	4.12 (3.21)
Product shape issue	3	14.68 (4.72–45.61)	14.68 (38.1)	14.63 (5.66)	3.87 (2.42)
Hair texture abnormal	45	14.6 (10.89–19.57)	14.55 (565.92)	14.5 (11.35)	3.86 (3.43)
Oncologic complication	3	14.13 (4.55–43.9)	14.13 (36.46)	14.08 (5.45)	3.82 (2.37)

Both ripretinib and sunitinib exhibited overlapping ADRs, predominantly palmar-plantar erythrodysesthesia syndrome (PPES), hyperkeratosis, and paradoxical disease progression. Mechanistic analysis suggests that PPES may originate from potent VEGFR signaling inhibition by both agents, leading to microvascular dysfunction and subsequent inflammatory tissue damage (Zhang et al., 2023). Notably, a multicenter phase III trial revealed significantly higher PPES incidence in the treatment group compared with placebo (12.5% vs. 0.8%), establishing it as the second most frequent ADR following hypertension (Lin et al., 2023), which corroborates our findings. The comparable hyperkeratosis rates observed with both TKIs imply a class effect potentially

mediated through EGFR or PDGFR pathway inhibition, which may disrupt keratinocyte proliferation and differentiation homeostasis (Malovitski et al., 2023). However, current pharmacovigilance data present an apparent discrepancy: while case report document ripretinib-associated hyperkeratosis (Muskat et al., 2022), no published clinical studies explicitly link sunitinib to this dermatological manifestation. This comparative analysis provides clinical evidence that hyperkeratosis represents a TKI-class adverse effect rather than a ripretinib-specific phenomenon. Of particular clinical significance was the identification of disease progression as an ADR, which likely reflects treatment failure mechanisms involving secondary kinase

TABLE 3 Top 30 AE analysis ranked by ROR value in sunitinib.

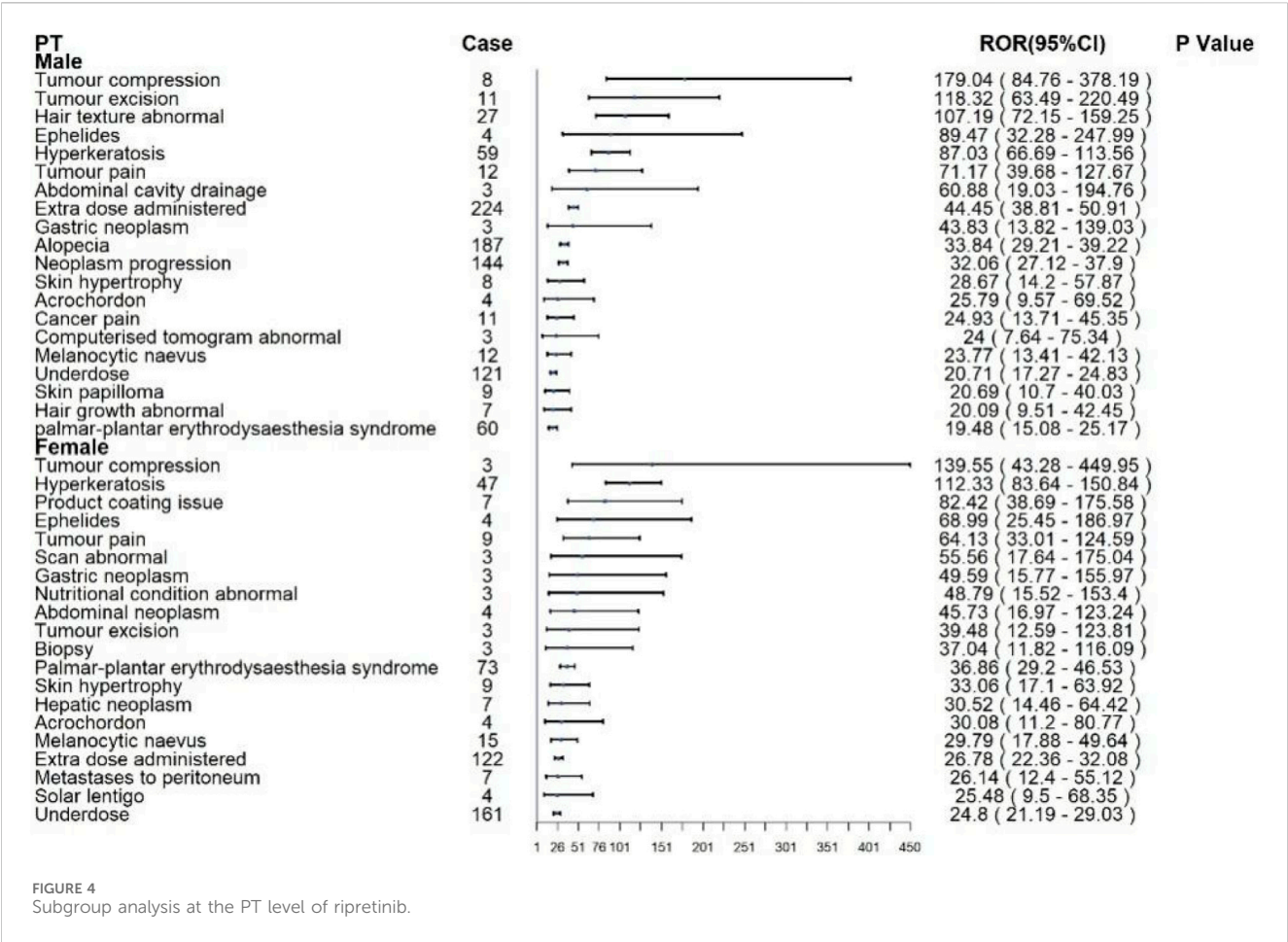
PT	N	ROR (95%CI)	PRR (χ^2)	EBGM(EBGM05)	IC(IC025)
Metastatic renal cell carcinoma	715	270.16 (245.71–297.04)	268.73 (114,239.39)	161.37 (149.05)	7.33 (7.21)
Diffuse uveal melanocytic proliferation	4	133.8 (43.15–414.88)	133.8 (395.42)	100.6 (39.03)	6.65 (5.18)
Renal cancer metastatic	179	127.34 (107.62–150.66)	127.17 (17,016.14)	96.81 (84.1)	6.6 (6.36)
Connective tissue neoplasm	6	109.48 (44.39–270.01)	109.47 (506.7)	86.23 (40.51)	6.43 (5.21)
Salivary gland fistula	3	109.48 (30.54–392.42)	109.47 (253.35)	86.23 (29.63)	6.43 (4.81)
Renal cell carcinoma stage iv	23	101.47 (64.22–160.33)	101.45 (1826.11)	81.19 (55.37)	6.34 (5.7)
Haemangiopericytoma	4	100.35 (33.55–300.18)	100.35 (314.75)	80.48 (32.18)	6.33 (4.89)
Renal cell carcinoma	896	98.62 (91.66–106.11)	97.97 (69,133.64)	78.95 (74.25)	6.3 (6.2)
Yellow skin	640	71.24 (65.49–77.5)	70.91 (37,490.48)	60.41 (56.3)	5.92 (5.79)
Malignant urinary tract neoplasm	16	69.82 (41.06–118.72)	69.81 (924.41)	59.62 (38.23)	5.9 (5.15)
Eyelash discolouration	29	65.78 (44.42–97.42)	65.77 (1589.28)	56.65 (40.78)	5.82 (5.26)
Gastrointestinal stromal tumour	312	57.76 (51.3–65.05)	57.63 (15,183.73)	50.52 (45.74)	5.66 (5.49)
Alveolar soft part sarcoma	4	47.23 (16.76–133.08)	47.22 (161.93)	42.36 (17.8)	5.4 (4.03)
Scrotal inflammation	5	40.96 (16.32–102.8)	40.96 (176.87)	37.26 (17.25)	5.22 (3.98)
Thyroid atrophy	6	40.82 (17.63–94.55)	40.82 (211.56)	37.14 (18.39)	5.22 (4.07)
Pancreatic neuroendocrine tumour	50	38.46 (28.77–51.41)	38.45 (1664.32)	35.17 (27.59)	5.14 (4.71)
Anal injury	13	35.99 (20.41–63.48)	35.99 (405.82)	33.11 (20.59)	5.05 (4.24)
Plantar erythema	17	32.19 (19.64–52.76)	32.19 (475.59)	29.87 (19.76)	4.9 (4.19)
Thymic cancer metastatic	3	31.69 (9.78–102.66)	31.69 (82.64)	29.44 (11.01)	4.88 (3.37)
Palmar-plantar erythrodysesthesia syndrome	1356	27.73 (26.23–29.3)	27.46 (32,368.06)	25.76 (24.6)	4.69 (4.61)
Pancreatic neuroendocrine Tumour metastatic	14	27.42 (15.95–47.11)	27.41 (333.51)	25.72 (16.35)	4.69 (3.92)
Myxoedema	12	26.47 (14.76–47.47)	26.47 (275.86)	24.89 (15.27)	4.64 (3.81)
Thymoma malignant	4	25.9 (9.42–71.19)	25.9 (89.94)	24.39 (10.47)	4.61 (3.27)
Neoplasm progression	1857	23.93 (22.83–25.09)	23.62 (38,009.99)	22.36 (21.5)	4.48 (4.41)
Mouth injury	78	23.9 (19.01–30.03)	23.88 (1614.08)	22.6 (18.66)	4.5 (4.16)
Tumour rupture	26	23.62 (15.9–35.08)	23.61 (531.74)	22.36 (16.06)	4.48 (3.91)
Oral pain	1071	22.19 (20.86–23.6)	22.02 (20,381.52)	20.93 (19.87)	4.39 (4.3)
Hyperkeratosis	221	18.58 (16.24–21.27)	18.55 (3508.64)	17.78 (15.88)	4.15 (3.95)
Cardiopulmonary failure	150	17.17 (14.58–20.22)	17.15 (2187.79)	16.49 (14.38)	4.04 (3.8)
Jaw fistula	5	16.87 (6.89–41.27)	16.87 (71.62)	16.23 (7.67)	4.02 (2.81)

mutations that confer therapeutic resistance or insufficient target inhibition. This paradoxical phenomenon underscores the need for molecular monitoring during TKI therapy escalation.

Subgroup analysis by gender offers critical insights into drug-specific ADRs. For ripretinib, both male and female patients exhibited elevated risks of tumor compression, hyperkeratosis, and tumor-related surgical interventions. Notably, female patients demonstrated higher incidences of tumor-associated pain and abnormal imaging findings, while males were more frequently affected by direct tumor-related complications, such as tumor excision and abdominal drainage. These disparities may reflect sex-specific pharmacokinetic and pharmacodynamic profiles,

including hormonal influences and differences in metabolic pathways. In contrast, gender differences in sunitinib-related ADRs were more pronounced. Male patients showed higher frequencies of metastatic renal cell carcinoma, salivary gland fistula, and eyelash discoloration, whereas females exhibited increased risks of renal cancer metastasis and yellow skin pigmentation. The ocular and cutaneous toxicities are likely attributable to sunitinib’s potent inhibition of VEGFR, which disrupts melanocyte function and melanin synthesis (Jin et al., 2023; Shah et al., 2021).

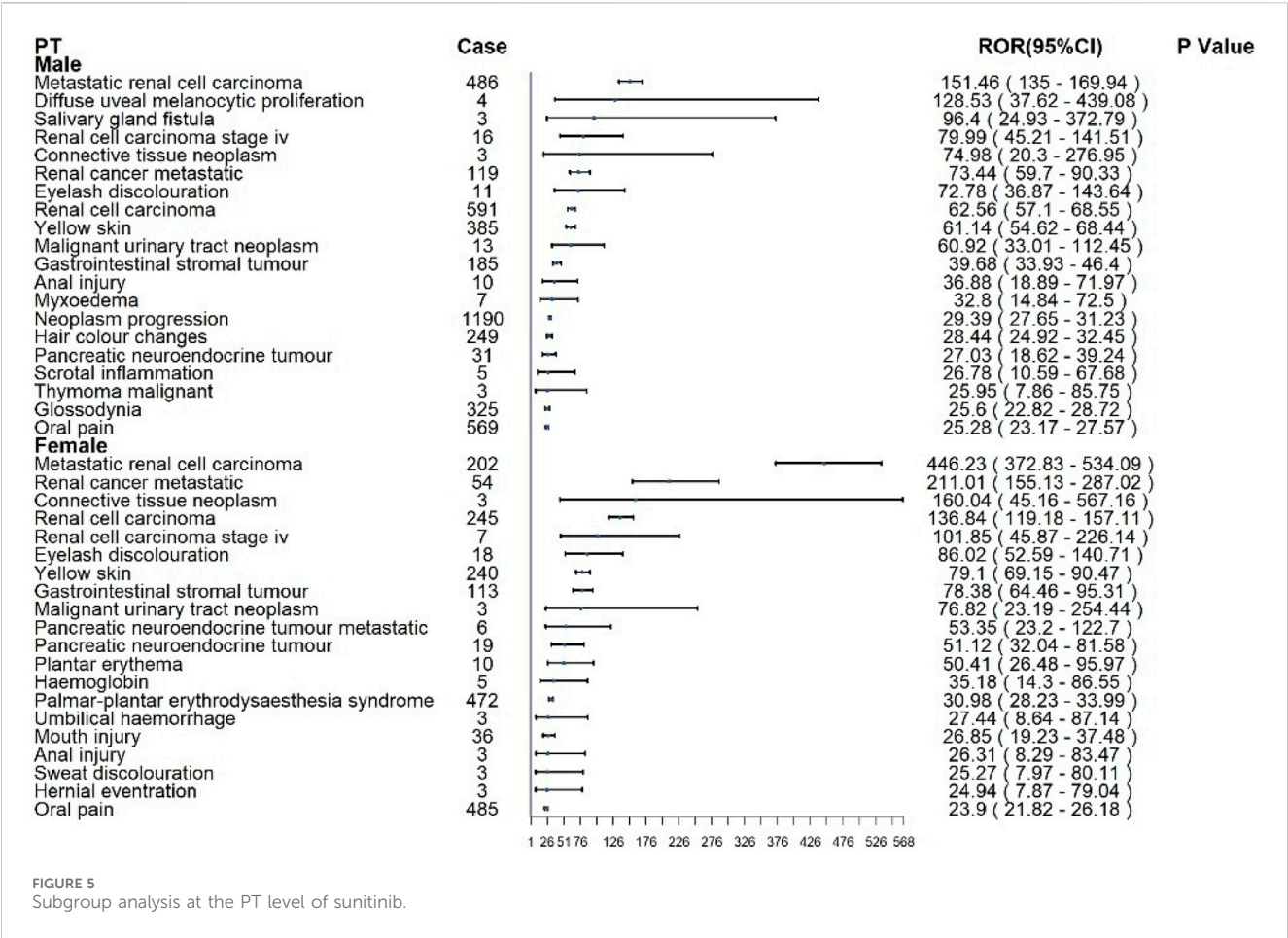
Despite these observations, research on sex-specific genetic mechanisms underlying ripretinib and sunitinib responses in



GIST remains limited. However, genomic analyses in clear cell renal cell carcinoma (CCRCC) have revealed that female patients with elevated *DKC1* expression also exhibit increased *TERC* levels, a pattern associated with reduced therapeutic response and shorter progression-free survival (Yuan et al., 2023). These findings suggest sex-dependent interactions between telomerase-related genes and tyrosine kinase inhibitor efficacy. Given the documented gender disparities in clinical ADRs among GIST patients—particularly cutaneous and ocular toxicities—comprehensive investigations into sex-specific molecular mechanisms are warranted. Key areas include kinase resistance pathways, telomerase regulatory networks, and polymorphisms affecting drug metabolism. Such research is essential to elucidate the biological underpinnings of therapeutic heterogeneity and to inform precision oncology through gender-stratified treatment strategies. A comparative mechanistic analysis further distinguishes ripretinib and sunitinib. Ripretinib is a novel type II switch control tyrosine kinase inhibitor designed to broadly inhibit both primary and secondary KIT and PDGFRA mutations associated with the progression of GIST (28, 29). Ripretinib exhibits high potency against the inactive form of receptor tyrosine kinases (RTKs) by binding to the switch pocket and activation loop, stabilizing the protein in an inactive conformation and thereby inhibiting its active state. This mechanism makes ripretinib a “switch control inhibitor,” with broad inhibitory activity against various secondary mutations, including KIT exons 13 (V654A), 14 (T670I), 17 (D816), and 18 (A829P) (Yu et al., 2021; Janku et al.,

2020). The drug was approved by the U.S. FDA in May 2020 for patients with advanced GIST who have failed at least three prior kinase inhibitors. Relevant studies support that ripretinib provides clinical benefits at a daily dose of 150mg, with better safety and patient tolerability compared to other treatments (Zalcberg).

Through a comprehensive analysis of ripretinib-related AEs in the FAERS database, we observed that the adverse signals associated with ripretinib primarily involved categories such as gastrointestinal disorders, general disorders and administration site reactions, and skin and subcutaneous tissue diseases. These results are consistent with the common adverse events reported in ripretinib’s drug labeling and clinical trials, such as nausea, vomiting, and fatigue (Dhillon, 2020). Ripretinib predominantly induces ADRs reflecting significant tumor volume changes and vascular alterations, such as hepatic embolization, which might be attributed to the profound vascular disruption caused by VEGFR pathway inhibition (Roskoski, 2021). Tumor compression, excision, and associated pain likely reflect the drug’s effective antitumor activity leading to rapid tumor cell necrosis, edema formation, and structural deformation, necessitating medical interventions (Schwartz, 2022; Lostes-Bardaji et al., 2021). Furthermore, this study identified some high-signal adverse events not mentioned in the drug’s prescribing information, such as skin papillomas, melanocytic nevi, and blood iron reduction. These new findings indicate that ripretinib may have potential effects on the skin, blood, and metabolism, which clinicians should monitor closely (Mühlenberg et al., 2024). In



addition, the study also identified signals for adverse events associated with severe outcomes. Among the reported serious adverse outcomes of ripretinib, 18.2% required hospitalization, and 10.6% involved deaths, some of which may be related to disease progression caused by the tumor. However, the possibility of severe reactions triggered by the drug itself cannot be ruled out. Notably, rare but high-signal adverse events, such as hepatic neoplasm, were reported, which are not explicitly described in the prescribing information. Their biological mechanisms and clinical significance require further research and validation.

Conversely, AEs of sunitinib prominently features distinct pathologic conditions such as diffuse uveal melanocytic proliferation, connective tissue neoplasm, and salivary gland fistula. Ocular adverse events associated with sunitinib therapy necessitate increased clinical vigilance. A case report described a male patient with renal cell carcinoma who developed bilateral diffuse uveal melanocytic proliferation (BDUMP) during sunitinib treatment, resulting in a fatal outcome (Parakh et al., 2022). BDUMP is a rare paraneoplastic ocular syndrome, typically occurring in patients with advanced, often occult, systemic malignancies (Tong et al., 2021). It serves as both a poor prognostic indicator and a marker of disease progression. The pathogenesis is hypothesized to involve ectopic production of growth factors or hormones that exert paracrine effects on distant ocular tissues. Experimental studies have demonstrated that IgG-enriched plasma fractions contain cultured melanocyte elongation and proliferation-stimulating factors (CMEP),

which induce abnormal proliferation of choroidal melanocytes and retinal pigment epithelial cells (Hu et al., 2023). These pathological changes disrupt retinal pigment epithelium function, ultimately compromising the outer blood-retinal barrier (Przeździecka-Dolyk et al., 2020; Sarkar et al., 2022). Additionally, unique ocular AEs like eyelash discoloration could result from disrupted melanocyte function and pigmentary alterations secondary to VEGFR inhibition.

Clinically, these differential AEs underscore the necessity of tailored patient risk assessment and individualized monitoring strategies. For ripretinib, clinicians should closely monitor hepatic function, tumor-related symptoms, and dermatologic health. For sunitinib, rigorous ophthalmologic evaluations, renal function monitoring, and attention to pigmentation changes are recommended, particularly in long-term therapy.

This study has several limitations. First, the FAERS database is a spontaneous reporting system that relies on voluntarily submitted adverse event reports. This reliance on spontaneous reporting may lead to underreporting of mild or common events or unusual events may be overreported. Second, the underlying conditions treated with certain drugs may predispose patients in GIST, acting as a confounding factor. Furthermore, establishing causality in pharmacovigilance and observational cohort studies is inherently challenging due to the lack of complete information in FAERS cases, such as dosage, frequency, duration of exposure, patient comorbidities, onset times, and other critical clinical details. This missing information limits the ability to fully analyze potential associations. Consequently, although we achieved a

comprehensive analysis of ripretinib's adverse reactions by removing indication restrictions, it should be noted that this generalized analytical approach may introduce data from non-target indications, potentially creating biases in identifying adverse reaction characteristics specific to GIST patients.

5 Conclusion

This study provides a comprehensive evaluation between ripretinib and sunitinib based on FAERS data. Known AEs were validated, and new potential safety signals were identified. The common AEs and differences between ripretinib and sunitinib should be taken into account to adjust clinical medication strategies. These findings contribute to the optimization of ripretinib and sunitinib's clinical use and emphasize the importance of continued pharmacovigilance.

Author contributions

XC: Writing – original draft, Conceptualization, Data curation, Formal Analysis. YZ: Funding acquisition, Supervision, Writing – review and editing, Resources, Validation, Visualization.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This research work was supported by the Traditional Chinese Medicine Science and Technology Project (Z-2023025) of the Shandong Provincial Health Commission.

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Acknowledgments

We extend our gratitude to the US FDA for providing free access to the data utilized in this study.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Correction note

A correction has been made to this article. Details can be found at: [10.3389/fphar.2025.1689499](https://doi.org/10.3389/fphar.2025.1689499).

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OPEN ACCESS

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RECEIVED 21 October 2024

ACCEPTED 13 June 2025

PUBLISHED 29 July 2025

CORRECTED 07 August 2025

CITATION

Zhang X, Jian T, Shi M, Wang C, Jian X and Li Q
(2025) An occupational pipe leak poisoning.
Front. Pharmacol. 16:1514565.
doi: 10.3389/fphar.2025.1514565

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An occupational pipe leak poisoning

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Introduction: Dimethylacetamide (DMAC) is used as a solvent in the dye, leather, and acrylic manufacturing industries. However, it is associated with significant risks of liver and nerve damage. 4,4'-Diaminodiphenyl ether (4,4'-ODA) is a crucial precursor for synthesizing curing agents and dyes but can disrupt the methemoglobin reduction system, resulting in methemoglobinemia.

Methods: This paper reports an incident involving a plant pipeline leak on May 10, 2024, which primarily contained a DMAC solution and trace amounts of 4,4'-ODA. The patient's occupational history, onset time of clinical symptoms, physical examination results, laboratory data, and treatment course were analyzed, and follow-up evaluation was performed to monitor the patient's long-term health status.

Results: Two patients exhibited clinical symptoms, including respiratory failure, methemoglobinemia, hemolytic anemia, and acute liver injury due to dermal and respiratory exposure. They were successfully treated and discharged. Notably, Patient 1 was diagnosed with toxic encephalopathy 16 days post-exposure, while Patient 2 developed peripheral neuropathy 58 days post-exposure.

Discussion: This study aimed to elucidate the clinical characteristics associated with this type of poisoning. Moreover, the incident calls for enhanced safety measures in industries using DMAC and 4,4'-ODA to prevent similar poisoning events.

KEYWORDS

dimethylacetamide, 4,4'-diaminodiphenyl ether, peripheral neuropathy, toxic encephalopathy, methemoglobinemia, toxic liver damage

1 Introduction

Dimethylacetamide (DMAC) (chemical formula, C₄H₉NO) is a colorless and transparent liquid that is soluble in both water and organic solvents such as benzene, alcohol, and ether. It is used as a solvent in various processes, including the production of synthetic resins and fibers (Nomiya et al., 2025). However, it can be absorbed through the skin and has significant irritative effects on the eyes, skin, and mucous membranes. It may exert detrimental effects on both the nervous system and hepatic function (Antoniou et al., 2021). Notably, no cases of toxic encephalopathy or peripheral neuropathy have been reported.

4,4'-Diaminodiphenyl ether (4,4'-ODA) (chemical formula, $C_{12}H_{12}N_2O$) is a white or light-yellow crystalline substance that exhibits slight solubility in water but dissolves readily in ethanol. It has an LD₅₀ value of 725 mg/kg for rats and is an aromatic amine compound (Singh et al., 2014). It is used as a crucial raw material for the synthesis of hardening agents and dyes. Exposure to 4,4'-ODA via inhalation or dermal absorption may lead to poisoning.

This study details an incident involving a pipeline leak at a factory on 10 May 2024, primarily containing DMAC along with potential residual amounts of 4,4'-ODA. Two patients were exposed through cutaneous contact and respiratory routes, resulting in methemoglobinemia accompanied by hemolytic anemia, acute liver injury, peripheral neuropathy, and toxic encephalopathy.

2 Clinical data

2.1 General patient information and factory production processes

Patient 1 was a 39-year-old man with 10 years of service. He had a history of good health prior to presentation. Patient 2 was a 64-year-old man with 16 years of service. He had a history of hypertension. They were an electric welder at the same leather factory. Both individuals were employed as electric welders at the same leather manufacturing facility, where they participated in pipe repair and equipment installation tasks. Their work environment typically involved outdoor settings without the use of protective gear such as hard hats, goggles, gloves, or respirators. They maintained an average work schedule of 8 hours per day for 28 days each month.

The factory produced 4,4'-ODA, which is used in the production of plastic films. The production process involved included the following: DMAC was added to the solution kettle to facilitate the reduction reaction of dinitrodiphenyl ether with hydrogen. Dinitrodiphenyl ether and activated carbon were introduced into the manhole, where the mixture was heated with steam and stirred. Once the dinitrodiphenyl ether was completely dissolved, the material was filtered into the reduction kettle using nitrogen pressure. After replacing the nitrogen, the reduction reaction was initiated by introducing hydrogen while agitating the mixture. After the reaction, the material was pressed through a filter using nitrogen. The filtrate was then transferred to the distillation kettle, where DMAC and water were distilled and condensed into a storage tank. The distillation residue was then pushed into the sublimation kettle with nitrogen, where the heating gas was condensed and crystallized in the crystallization chamber to yield crude 4,4'-ODA. Additionally, the event pipeline stored the DMAC solution for recycling after the reaction, which may also contain residue from 4,4'-ODA. The pipeline of this equipment had been used for 6 years without repair. The waste liquid had also been stored in the pipeline.

2.2 Poisoning incidents and treatment

At 8 a.m. on 10 May 2024, the two patients were conducting pipeline maintenance in the old factory area. Upon opening the blue

sheet of the pipeline, a brown-red liquid containing rust sprayed out, accompanied by a pungent odor. The liquid sprayed onto the patients' bodies, affecting their hair, face, eyes, and other areas. Both patients reported a burning sensation and observed blackened skin where the liquid had contacted them. They were washed after 20 min of exposure.

2.2.1 Patient 1

On the way to the hospital (approximately 40 min after the incident), Patient 1 experienced dizziness, confusion, shallow breathing, limb twitches, and dyspnea, with symptoms gradually worsening. Upon admission, he was delirious and comatose. His pupils were large and round, measuring approximately 3 mm in diameter, with a slow light reflex. Cyanosis was observed in the skin of his face, lips, extremities, and nail beds. His heart rate was 100 beats/min and regular, with no pathological murmurs detected in any valve area. His blood pressure was 88/48 mmHg, and his respiratory sounds were thick in both lungs, with no dry rales present. The arterial blood gas analysis revealed the following: pH, 7.382 (7.350–7.450); lactic acid, 2.96 mmol/L (0.50–1.70); pCO₂, 37.7 mmHg (35.0–45.0); pO₂, 61 mmHg (80–105); and methemoglobin, 43.6%. Immediate treatment included tracheal intubation and ventilator-assisted ventilation. Three hours later, a repeat arterial blood gas analysis revealed the following: pH, 7.294 (7.350–7.450); and lactic acid, 10.48 mmol/L (0.50–1.70). The patient received intravenous injections of methylene blue (20 mg, twice a day) and dexamethasone (10 mg), along with liver protection and blood purification treatments. The laboratory test results for Patient 1 are presented in Table 1. On May 11, the toxicology test indicated a DMAC concentration of 0.530 µg/mL in the blood (detection was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS)). The patient developed hemolytic anemia, with significantly increased white blood cell count, liver function markers, and creatine kinase levels, all of which progressively worsened. The patient was transferred to our hospital on the sixth day after the poisoning for further diagnosis and treatment.

Upon admission, he was sedated and on ventilator-assisted ventilation, displaying uncooperative behavior during the physical examination. He had yellowing of the skin and sclera, and his pupils were large and round, measuring about 3 mm in diameter with a dull light reflex. His heart rate was 84 beats/min, consistent and rhythmic, and his body temperature was 38.7°C. His urine was soy sauce-colored (Figure 1), with a volume of 1,200 mL. The arterial blood gas analysis revealed the following: pH, 7.527 (7.350–7.450); lactic acid, 2.1 mmol/L (1.0–1.8); and methemoglobin, 1.0% (0–3). His hemoglobin level was 62.0 g/L (130–175). The patient received two units of red blood cells, along with dexamethasone (10 mg), furosemide (20 mg), vitamin B₁ (100 mg), as well as liver protective agents (polyene phosphatidylcholine), nerve nutrition, and blood purification treatments. On the seventh day after poisoning, the patient experienced sporadic limb convulsions, with the urine volume increasing to 1900 mL and its color intensity decreasing. Valproate was administered to control the convulsions, and two units of red blood cells were transfused alongside continued blood purification. On the ninth day, the patient's consciousness improved, and the urine color returned to normal. By the 11th day, the patient's

TABLE 1 Laboratory test results of the patient 1 at different time-points following hospital admission.

Biochemical blood indicators	Local hospital							Our department						
	Normal values	Day1	Day2	Day3	Day4	Day5	Day6	Normal values	Day6	Day8	Day12	Day19	Day28	Day60
WBC($\times 10^9/L$)	3.5–9.5	23.85	27.07	25.98	28.12	24.30	33.55	3.5–9.5	29.23	31.33	27.27	6.83	5.14	7.73
NEU(%)	40–75	99.8	90.9	87.8	86.4	—	83.2	40–75	86.10	86.30	85.40	71.50	56.60	59.70
ALT (IU/L)	0–41	53	50	35	27	—	37	0–35	43	37	76	147	54	38
AST (IU/L)	0–37	29	52	40	26	—	23	14–36	68	31	28	36	21	21
TBIL ($\mu\text{mol/L}$)	5.1–19	32.59	30.18	43.87	57.19	58.42	84.85	3–22	89	47.5	22.3	5.9	7.0	7.0
DBIL ($\mu\text{mol/L}$)	1.7–6.8	15.08	13.72	17.65	20.86	23.28	27.84	0–5	1	17.8	9.3	3.3	3.1	2.4
IBIL ($\mu\text{mol/L}$)	2–22	17.51	16.46	26.22	36.33	35.14	57.01	0–19	71	29.7	13.0	2.6	3.9	4.7
CK(U/L)	24–190	257.6	1,260	884.6	476.4	—	195.6	30–135	144	113	56	27	33	47
CK-MB(ng/mL)	0–24	20	41	28	16	—	9	0.3–4	1.20	0.5	1.8	1.9	0.9	0.8
BUN(mmol/L)	1.7–8.3	4.4	4.6	4.9	6.6	7.0	8.2	2.5–6.1	9.8	11.32	8.56	2.83	3.23	3.10
Cr ($\mu\text{mol/L}$)	70–115	88	76	76	63	54	62	46–106	73	82	61	65	76	87
IL-6 (pg/mL)	—	—	—	—	—	—	—	0–7	—	24.60	6.09	—	<2.00	—
RBC($\times 10^{12}/L$)	4.3–5.8	5.35	5.47	4.32	—	2.98	2.01	4.3–5.8	1.17	1.72	3.13	3.06	4.11	5.29
HGB (g/L)	130–175	158	156	122	—	82	75	130–175	62.0	59.0	103.0	99.0	129.0	154.0
DD-i ($\mu\text{ g/mL}$)	—	—	—	—	—	—	—	<0.50	2.99	5.56	5.16	1.00	0.72	0.36

WBC, white blood cells; NEU, neutrophils; ALT, alanine transaminase; AST, aspartate aminotransferase; TBIL, total bilirubin; DBIL; direct bilirubin; IBIL; indirect bilirubin; CK, creatine kinase; CK-MB, creatine kinase-MB; BUN, blood urea nitrogen; Cr, creatinine; IL-6, interleukin 6; RBC, red blood cells; HGB, hemoglobin; DD-i, D-dimer.

Values in bold represent the normal values of indicators for different hospitals.



FIGURE 1
On the sixth day post-exposure to the toxins, the urine of Patient 1 exhibited a coloration similar to that of soy sauce.

condition had improved significantly. The tracheal tube was removed, and the yellowing of the sclera and skin was alleviated. On the 13th day, a chest computed tomography (CT) revealed a small amount of inflammatory changes in both lungs, fibroproliferative foci, and reduced density in the heart cavity, indicating anemia. No significant abnormalities were observed in the head and abdominal CT scans. However, the liver function markers remained elevated, prompting continued liver preservation therapy. On the 16th day, the patient reported numbness and weakness in his fingertips. Cerebral magnetic resonance imaging (MRI) revealed bilateral cerebral hemispheres under the cortex, and the internal sac showed symmetrical distribution of long T_1 and T_2 signals. The T_2 FLAIR sequence demonstrated hyperintensity, while diffusion-weighted imaging (DWI) indicated high signal intensity alongside multiple abnormal signals observed within the brain, which was consistent with the manifestations of toxic encephalopathy (Figure 2). The patient received vitamin B₁, mecobalamine, and other nerve nutrition treatments. By the 21st day, the patient's condition had improved, and he was discharged from the hospital. On the 28th day, a follow-up chest CT showed improvement in the symptoms, and the range of abnormal cranial magnetic signals had reduced. However, on the 60th day of re-examination, the family reported decreased speech, and he reported persistent numbness and weakness in the left thumb. The cranial MRI revealed multiple abnormal signals in the brain (Figure 3). A few symmetrically distributed long T_1 and T_2 signals were observed in the white matter and basal ganglia areas of the bilateral cerebral hemispheres. The electromyography

and electroencephalogram results showed no obvious abnormalities, and we continued to provide nutritional nerve therapy and other symptomatic treatments. Regular reviews indicated no obvious discomfort.

2.2.2 Patient 2

On the way to the hospital, Patient 2 developed chest tightness, dyspnea, cyanosis of the mouth, and a pale face, with the symptoms progressively worsening. Upon admission, the patient exhibited a dull response, shallow breathing, and vague consciousness. His pupils were large and round, measuring approximately 3 mm in diameter, with a dull light reflex. His heart rate was 60 beats/min, and no pathological murmurs were detected in any valve area. His blood pressure was 120/70 mmHg. Moreover, the arterial blood gas analysis revealed the following: pH, 7.334 (7.350–7.450); lactic acid, 1.32 mmol/L (0.50–1.70); methemoglobin, 38.6%. No significant abnormalities were found in the patient's brain and chest CT scans on the first day of poisoning. Moreover, symptomatic treatments, including tracheal intubation, ventilator-assisted ventilation, sedation, and intravenous methylene blue injection (20 mg twice daily), along with blood purification, were administered at a local hospital. After treatment, the patient's consciousness improved. The laboratory test results of Patient 2 are presented in Table 2. On May 11, the toxicology test indicated a DMAC concentration of 0.588 $\mu\text{g/mL}$ in the blood (detection was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS)). The patient also developed hemolytic anemia and liver function impairment. Tracheal intubation was discontinued on the fourth day after poisoning. CT revealed increased fibrous foci in both lungs and new hypostatic inflammation, with no brain abnormalities. He was admitted to our hospital on the seventh day for further treatment.

Upon admission, the patient was conscious and cooperative during the physical examination. He exhibited slight yellowing of the skin and mucous membranes, with his pupils measuring approximately 3 mm in diameter with a normal light reflex. His heart rate was 82 beats/min. He was treated with dexamethasone (10 mg), vitamin B₁, mecobalamine, liver protective agents (polyene phosphatidylcholine), and blood purification. On the 12th day after poisoning, chest CT revealed inflammation in the lower lobes of both lungs. The cerebral MRI revealed slight ischemic degeneration of white matter in both cerebral hemispheres. On the 60th day, follow-up MRI and CT scans showed no significant abnormalities. The electroencephalogram was normal, but electromyography indicated peripheral neuropathy in the upper and lower extremities, primarily affecting motor and sensory functions. Notable findings included decreased conduction velocity in the median motor nerve at the cubital tunnel, reduced amplitude in the left superficial peroneal nerve conduction, absent conduction in the right superficial peroneal nerve, decreased conduction velocity in the left sural nerve, and absent conduction in the left tibial nerve. The patient continued to receive vitamin B₁, mecobalamine, and other nutritional nerve treatments, with regular follow-up reviews.

3 Discussion

The poisoning substances primarily involved DMAC and 4,4'-ODA, which have not been previously reported. When the patients

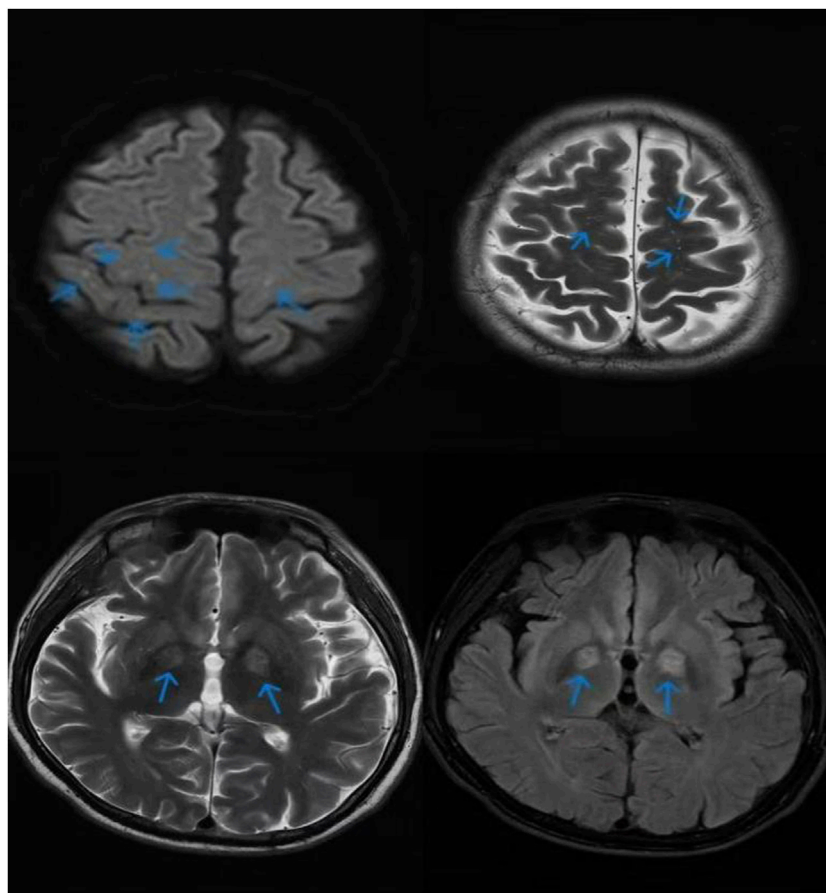


FIGURE 2

The cranial magnetic resonance imaging (MRI) result of Patient 1 revealed bilateral cerebral hemispheres beneath the cortex, with the internal capsule exhibiting a symmetrical distribution of spots and dots characterized by prolonged T1 and T2 signals. The T2 FLAIR sequence demonstrated hyperintensity, while diffusion-weighted imaging (DWI) indicated high signal intensity alongside multiple abnormal signals observed within the brain.

opened the blue sheet of the pipeline, a waste liquid stored in the pipeline at normal temperature was ejected. Following transient exposure to high concentrations of the toxins, the patients exhibited dizziness, unconsciousness, cyanosis of the lips, nail beds, and skin, as well as progressing to a coma. Laboratory examinations showed methemoglobinemia and liver function damage. We speculate that 4,4'-ODA, an aromatic amine compound, was responsible for the methemoglobinemia (National Toxicology Program, 1980). It can cause poisoning through inhalation and skin contact and is known to have irritating, mutagenic, and carcinogenic effects (Hayden et al., 1978). In the body, it oxidizes to produce toxic metabolites. 4,4'-ODA binds to hemoglobin as a diamine or monoacetyl-diamine (Sabbioni and Schütze, 1998). It can lead to acquired methemoglobinemia. The direct oxidation of hemoglobin in the blood to methemoglobin may also interfere with the methemoglobin reduction system in red blood cells (Coleman et al., 1991), resulting in tissue hypoxia, diffuse cyanosis, and hemolytic anemia.

Acquired methemoglobinemia is most commonly caused by various chemical substances or drug poisoning (Dhungel et al., 2021). In this condition, the bivalent iron in the hemoglobin molecule is replaced by trivalent iron, resulting in a loss of the ability to bind oxygen. Common toxic agents include sodium nitrite,

aniline, and nitrobenzene. Typically, when methemoglobin concentrations exceed 50%, severe symptoms, such as shock, convulsions, and coma, may manifest. Methylene blue is the primary treatment for reducing methemoglobin levels (Iolascon et al., 2021). Hemolysis associated with methemoglobinemia can be attributed to the toxicity of substances, G6PD deficiency (Schoorman et al., 2009), and methylene blue overdose. In this event, the poison or its metabolites can cause hemolysis by damaging cell membranes or disrupting intracellular enzyme systems (Yu et al., 2022; Shi et al., 2022), resulting in a rapid decline in red blood cell count and hemoglobin levels, leading to hemolytic anemia, alongside significant increases in bilirubin levels and liver function impairment. In Patient 1, hemolytic anemia was particularly severe, and muscle tissue injury was exacerbated by convulsions, resulting in a significant increase in creatine kinase levels. The patient's condition improved after transfusion, blood purification, and symptomatic treatment. Studies have demonstrated that blood purification can significantly decrease creatine kinase levels in the bloodstream, consequently safeguarding renal function.

In addition, subsequent to discharge, all patients exhibited neurological deficits. Patient 1 exhibited fingertip numbness

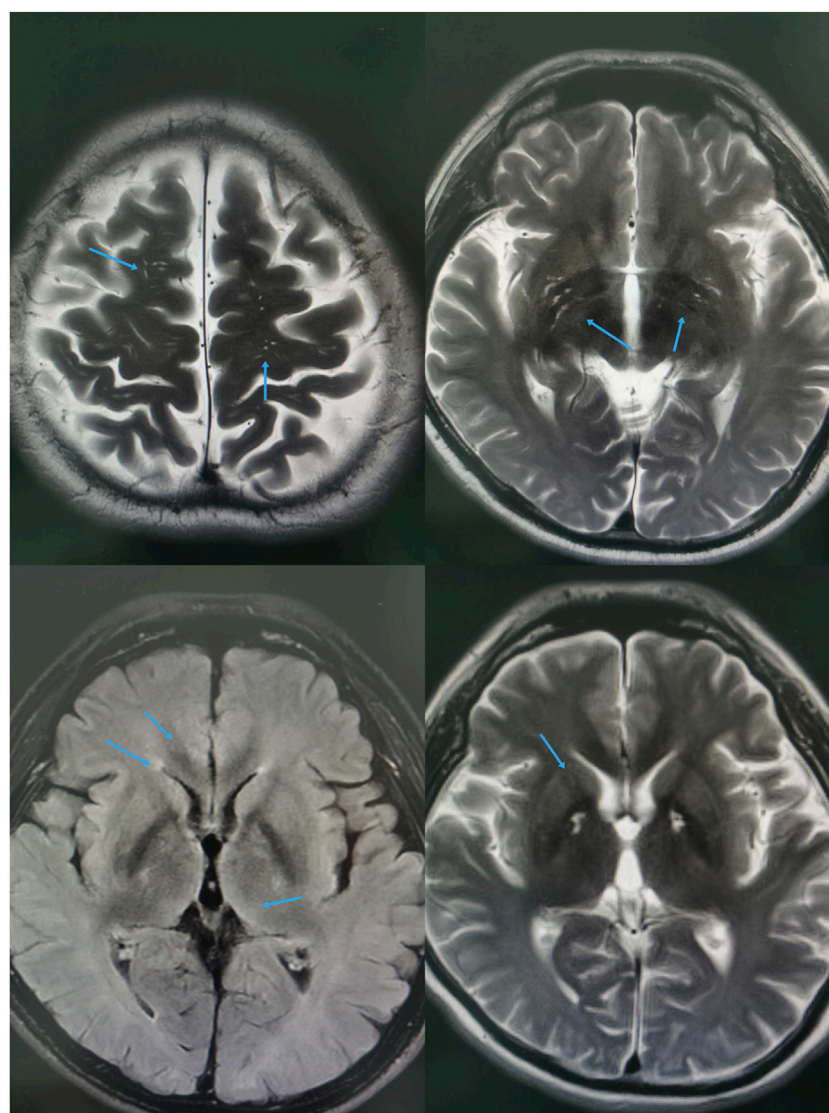


FIGURE 3

The MRI revealed white matter abnormalities in both cerebral hemispheres, with several long T₁ and T₂ signal lesions exhibiting symmetrical distribution within the basal ganglia. The T₂ FLAIR sequence demonstrated hyperintensities, while diffusion-weighted imaging (DWI) indicated mildly elevated signals, alongside multiple abnormal findings throughout the brain.

16 days after poisoning. The MRI findings revealed a symmetrical distribution of speckle-like and punctate-long T₁ and T₂ signals within the internal capsule beneath the cortex of both cerebral hemispheres. T₂ FLAIR revealed hyperintensity, while DWI indicated elevated signals consistent with toxic encephalopathy. Abnormal signals in the internal capsule have been more frequently observed in patients suffering from carbon monoxide poisoning, heroin encephalopathy, and toluene toxicity. Patient 2 developed peripheral neuropathy 60 days after exposure, predominantly manifesting as motor and sensory deficits. Nevertheless, the exact pathophysiological mechanisms remain elusive. Notably, there is no prior documentation of toxic encephalopathy or peripheral nervous system damage resulting from DMAC and 4,4'-ODA poisoning.

Notably, the *in vitro* cytotoxicity experiments conducted on Hep 3B and IMR 32 human cell lines demonstrated that 4,4'-ODA is

capable of inducing apoptosis (Singh et al., 2014). Cases of DMAC poisoning are predominantly reported following prolonged chronic exposure or high-dose incidents, often presenting with significant hepatitis (Wang and Chen, 2020), gastrointestinal symptoms, and central nervous system damage within a 2-month incubation period. Patients with acute severe toxic hepatitis induced by DMAC may experience relapses over short intervals, with liver enzyme levels typically rising. Furthermore, abnormal liver function, accompanied by pathological alterations, often leads to disrupted bilirubin metabolism and jaundice. Dimethylacetamide is mainly metabolized by cytochrome P450 in the liver, and its hepatotoxicity is associated with ROS-induced DNA damage (Perbellini et al., 2003). Neurotoxicity primarily affects the central nervous system. Workers subjected to long-term DMAC exposure frequently report symptoms such as headaches, vertigo, lethargy, hallucinations, depression, and impairments in speech and

TABLE 2 Laboratory test results of the patient 2 at different time-points following hospital admission.

Biochemical blood indicators	Local hospital				Our department							
	Normal values	Day4	Day5	Day6	Normal values	Day6	Day8	Day12	Day19	Day28	Day60	Day67
WBC (×10 ⁹ /L)	3.5–9.5	14.18	14.23	24.30	3.5–9.5	13.63	10.62	15.57	8.49	6.74	7.01	6.21
NEU (%)	40–75	90.0	88.5		40–75	83.50	77.80	85.40	66.10	65.50	52.10	61.00
ALT (IU/L)	0–41	117	157	—	0–35	147	117	85	32	16	14	12
AST (IU/L)	0–37	89	84	—	14–36	64	49	29	16	17	19	17
TBIL(μmol/L)	5.1–19	21.36	66.64	58.42	3–22	40	30.7	12.19	10.8	6.4	7.3	7.0
DBIL (μmol/L)	1.7–6.8	9.53	38.18	23.28	0–5	0	12.4	5.5	4.2	3.2	2.6	2.3
IBIL (μmol/L)	2–22	11.83	28.46	35.14	0–19	28	18.3	7.4	6.6	3.2	4.7	4.7
CK (U/L)	24–190	122.4	107.2	—	30–135	136	129	46	27	48	68	86
CK-MB(ng/mL)	0–24	13	16–	—	0.3–4	1.90	0.8	0.5	0.6	0.6	0.9	0.9
BUN (mmol/L)	1.7–8.3	5.6	11.7	7.0	2.5–6.1	9.1	9.56	7.63	5.76	6.11	5.39	4.22
Cr (μmol/L)	70–115	51	79	54	46–106	68	69	61	72	79	83	75
IL-6(pg/mL)	—	—	—	—	0–7	16.9	3.56	3.46	—	2.20	—	
RBC(×10 ¹² /L)	4.3–5.8	3.57	3.53	2.98	4.3–5.8	2.99	2.92	2.70	3.25	3.40	4.46	4.17
HGB(g/L)	130–175	107	105	82	130–175	94.0	91.0	84	105.0	110.0	134.0	126.0
DD-i (μ g/mL)	0–1	11.66	—	—	<0.50	1.95	0.90	1.12	1.19	0.91	0.38	0.63

WBC, white blood cells; NEU, neutrophils; ALT, alanine transaminase; AST, aspartate aminotransferase; TBIL, total bilirubin; DBIL, direct bilirubin; IBIL, indirect bilirubin; CK, creatine kinase; CK-MB, creatine kinase-MB; BUN, blood urea nitrogen; Cr, creatinine; IL-6, interleukin 6; RBC, red blood cells; HGB, hemoglobin; DD-i, D-dimer.
Values in bold represent the normal values of indicators for different hospitals.

cognition, which can even progress to coma (Song et al., 2018; Tutkun et al., 2019). These effects may be linked to oxidative stress responses, lipid peroxidation, calcium homeostasis disruption, and apoptosis of target cells, among other factors (Liu et al., 2016).

4 Conclusion

In conclusion, DMAC and 4,4'-ODA can be absorbed via the respiratory tract and skin, leading to potential damage to the liver and kidneys, as well as the nervous system. Early and appropriate interventions, such as hemoperfusion and blood purification, should be implemented following diagnosis (Su et al., 2000). Moreover, monitoring the progression of hemolytic anemia and hepatic injury is essential. Measures should be taken to mitigate intracranial edema, alleviate clinical symptoms (Dang et al., 2019), prevent toxic encephalopathy and peripheral nerve damage.

5 Recommendations

Our case underscores the necessity for enterprises that employ workers in contact with 4,4'-ODA and DMAC to provide comprehensive occupational health training, enhance awareness of the toxicity of these substances, reinforce safety protocols, and conduct regular health assessments (Li et al., 2022). Encourage enterprises to strengthen preventive measures to reduce the risk of occupational environmental exposure to poisons. For example, in

2024 the JSOH(Japanese Society of Occupational Health) recommended 5 ppm as the occupational exposure limit (OEL) for DMAC. These measures can help prevent the recurrence of similar poisoning incidents.

6 Limitations

Limitations of the data presented here, such as the lack of controlled variables or well-defined baselines for assessing the severity of outcomes, will be refined in future work.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Ethics Committee of Shandong University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the

publication of any potentially identifiable images or data included in this article.

Author contributions

XZ: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Writing – original draft. TJ: Conceptualization, Data curation, Investigation, Writing – original draft. MS: Conceptualization, Formal Analysis, Investigation, Writing – original draft. CW: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft. XJ: Investigation, Resources, Supervision, Validation, Writing – review and editing. QL: Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Validation, Visualization, Writing – review and editing.

Funding

The author(s) declare that no financial support was received for the research and/or publication of this article.

Acknowledgments

We thank the staff of Qilu Hospital of Shandong University for their support with sample and clinical data collection.

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Correction note

This article has been corrected with minor changes. These changes do not impact the scientific content of the article.

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RECEIVED 03 January 2025

ACCEPTED 10 July 2025

PUBLISHED 30 July 2025

CITATION

Ren Z-J, Zhang Q, Tang N-X, Li Y-D, Lu D-L,
Lin A-L, Yang C and Wang F (2025)
Environmental cadmium exposure and the
risk of kidney stones: a systematic review and
dose-response meta-analysis.
Front. Med. 12:1555028.
doi: 10.3389/fmed.2025.1555028

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Environmental cadmium exposure and the risk of kidney stones: a systematic review and dose-response meta-analysis

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Background: Recent studies have investigated the relationship between cadmium exposure and kidney stones. Nevertheless, the results remain controversial. Therefore, we performed a comprehensive systematic review and meta-analysis based on the latest evidence to address gaps in the research.

Methods: Medline, Embase, and the Web of Science databases were searched to identify relevant studies up until 31 July 2024. Characteristics and outcomes of the included studies were extracted following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines and the Meta-analyses of Observational Studies in Epidemiology (MOOSE) guidelines. A random effects model was used to determine the association between cadmium exposure and the risk of kidney stones.

Results: A total of 17 studies involving 159,011 individuals were included in the meta-analysis. When comparing the highest versus lowest cadmium exposure levels, the overall relative risk (RR) for kidney stones was 1.19 [95% confidence interval (CI): 1.10–1.29]. Subgroup analysis showed that urinary (RR = 1.19; 95%CI: 1.08–1.30) and blood (RR = 1.49; 95% CI: 1.10–2.02) cadmium levels were associated with an increased risk of kidney stones. In contrast to non-cadmium-contaminated areas, both blood (RR = 1.08; 95% CI: 1.00–1.15) and urinary (RR = 1.16; 95% CI: 1.05–1.27) cadmium levels were associated with an increased risk of kidney stones in cadmium-contaminated areas. In the dose-response meta-analysis, we observed a consistent linear positive association between cadmium exposure and the risk of kidney stones. The overall RR for every 1.0 µg/L increase in urinary cadmium levels was 1.07 (95% CI: 1.01–1.13).

Conclusion: Our findings suggest that cadmium exposure is associated with the risk of kidney stones. These findings reinforce the importance of environmental cadmium exposure as a risk factor for kidney stones, extending beyond the influence of conventional risk factors. Efforts to reduce cadmium exposure in the population may help reduce the individual, economic, and societal burdens associated with kidney stones.

Systematic review registration: <https://www.crd.york.ac.uk/PROSPERO/myprospéro>.

KEYWORDS

kidney stones, cadmium exposure, heavy metal, systematic review, meta-analysis

Introduction

Kidney stones are a common condition worldwide, with incidence and prevalence increasing among both children and adults (1–3). Approximately 10 ~ 12% of men and 5 ~ 6% of women are affected by kidney stones (4). Kidney stone formation is a complex process resulting from an imbalance in urine between promoters and inhibitors of crystal formation (5, 6). Various risk factors, such as geography, diet, genetics, and occupation, can affect this balance, resulting in the development of kidney stones (7). Among these risk factors, environmental factors are recognized as important contributors to kidney stone formation (8, 9).

Cadmium, a heavy metal, is one of the most toxic industrial and environmental pollutants, which poses a severe threat to human health (10, 11). Cadmium can enter the body through air, water, soil, and food, and it largely accumulates in the kidneys, liver, bones, and other organs, causing irreversible damage to the target organs (12–14). Previous studies have evaluated different types of heavy metals and their concentrations in urinary stones (15, 16). Notably, significantly higher concentrations of 17 elements, including cadmium, were found in all types of stones (15, 16). In addition, cadmium concentrations were higher in calcium phosphate stones, along with other elements (15). Previous studies have reported that cadmium accumulation causes cellular toxicity and damages multiple organs (17). Long-term exposure to cadmium has been linked to a higher calcium excretion rate and tubular impairment with a loss of reabsorptive capacity, which increase the risk of kidney stone formation (18, 19).

Several observational studies have attempted to address the association between cadmium exposure and the risk of kidney stones. However, their results remain controversial. To better understand this issue, we conducted a comprehensive systematic review and meta-analysis of published literature that investigated the correlation between cadmium exposure and the risk of kidney stones.

Methods

Literature search and eligibility criteria

Medline, Embase, and the Web of Science databases were searched up until 31 July 2024. The search terms included “metal exposure OR cadmium” and “kidney stones OR nephrolithiasis OR urolithiasis OR renal stones.” The reference lists of relevant studies were reviewed to identify additional studies. Figure 1 shows the search strategy. Studies were considered eligible if they (1) were published in the English language; (2) had the full text available; (3) evaluated the relationship between cadmium exposure and the risk of kidney stones; (4) provided risk estimates with confidence intervals (CIs) or offered data to calculate these associations; and (5) were case-control, cohort, or cross-sectional studies. The protocol was registered with PROSPERO (CRD42024564167) on 11 July 2024.

Data extraction

Data were independently extracted by two investigators using a standardized collection form. The relevant data extracted included the following: first author, publication date, study design, study

region, sample size, effect estimates [OR, relative risk (RR), HR, or IRR] with 95% CIs, and the potential confounders used for adjustment. Any discrepancies in the results were resolved through discussion with a third investigator.

Quality assessment

Two investigators used the Newcastle–Ottawa scale (NOS) to conduct quality assessments of case-control and cohort studies (20). A maximum of 9 stars are awarded to each study based on three aspects: 4 stars for the selection of participants, 2 stars for the comparability of groups, and 3 stars for the assessments of outcomes. Scores of 7–9, 4–6, and 0–3 were categorized as high, moderate, and low quality, respectively, for each study. The quality assessment of the cross-sectional studies was conducted following the guidelines provided by the Agency for Healthcare Research and Quality (21). A total of 11 items were included in this self-rating scale, with each item worth one point. The following score categories were used to assess article quality: 0–3 indicated low quality, 4–7 indicated moderate quality, and 8–11 indicated high quality.

Statistical analysis

The primary outcome was the relative risk of kidney stone incidence. Subgroup analyses of the primary outcome were conducted based on sex and whether the area was cadmium-contaminated. For each study, the risk ratio for kidney stones with the corresponding 95% CI was calculated. A random effects model was used to compute the pooled risk ratio. A chi-squared-based Q test and the I^2 statistic were performed to evaluate the heterogeneity between studies. If I^2 is greater than 50% and the p -value is less than 0.10, heterogeneity was considered statistically significant. A Z-test was performed to assess the significance of the overall RR, and a p -value of < 0.05 was considered statistically significant. We carried out a dose-response meta-analysis of the risk of kidney stones according to the methods proposed by Orsini et al. (22) and Berlin et al. (23). Each category's mean concentration of cadmium was taken as the corresponding dose. When the upper boundary of the highest category was open-ended, the midpoint was calculated by multiplying the lower boundary by 1.5. We set the lowest category to zero, if it was unavailable. The linear and non-linear models were evaluated based on the null hypothesis, with the spline coefficients set to zero. A sensitivity analysis was conducted to evaluate the stability of the results by excluding one study at a time. Potential publication bias was tested using funnel plots, Begg's test, and Egger's test. All statistical analyses were conducted using Stata software (version 14.0) (Stata Corporation, College Station, Texas, United States).

Results

Search results and study characteristics

The systematic search of articles published up to 31 July 2024 identified 408 articles. After screening the titles and abstracts,

we obtained 42 studies for a full-text review. After the full-text review, we finally included 17 published studies comprising 159,011 individuals in the analysis (19, 24–39) (Figure 1). Among these, 1 study was a cohort study, 3 were case–control studies, and 13 were cross-sectional studies. Six of these studies were conducted in America, five in Europe, and six in Asia. Furthermore, 14 studies reported cadmium concentrations measured in urine, 5 studies reported cadmium concentrations measured in blood, and 1 study reported cadmium concentrations measured in dietary sources. The articles were published between 1985 and 2024. The detailed characteristics of all included studies are shown in Table 1. The

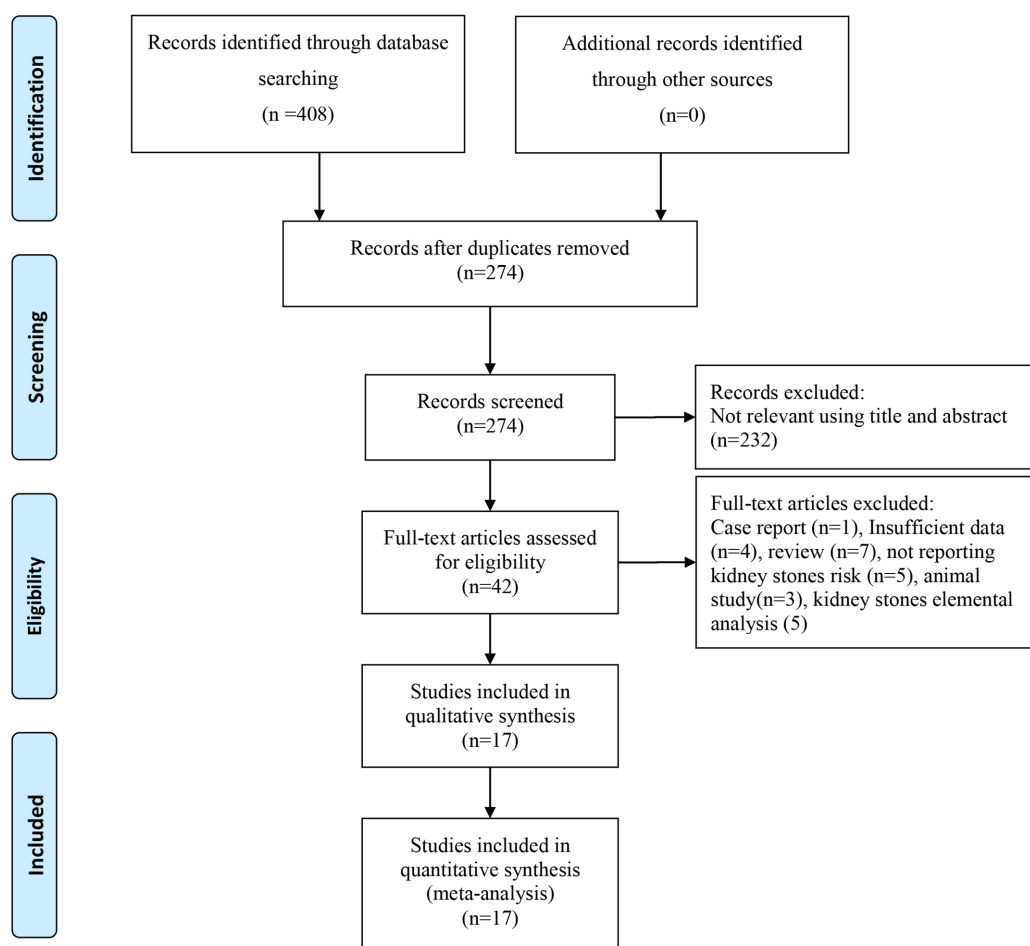
majority of studies were of medium to high quality. One cross-sectional study was of low quality (Table 1).

Quantitative synthesis

A total of 17 studies involving 159,011 individuals evaluated the association between cadmium exposure and the risk of kidney stones. The results showed that cadmium exposure was associated with an increased risk of kidney stones ($RR = 1.19$; 95% CI: 1.10–1.29, Figure 2). Significant heterogeneity was observed among the



PRISMA 2009 Flow Diagram



From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

For more information, visit www.prisma-statement.org.

FIGURE 1
PRISMA flow diagram illustrating the selection of the included studies.

TABLE 1 Study population and exposure characteristics of the studies included in the meta-analyses.

Author (year)	Country	Study design	Study quality	Sample size	Biological sample type	Measure of effect	RR (kidney stone risk) (95% CI)	Adjustment factors
Lu et al. (2024) (39)	United States	Cross-sectional study	High	8,515	Urine	OR	1.663 (1.277, 2.167)	Age, sex, ethnicity, education levels, marital status, BMI, hypertension, diabetes, vigorous recreational activities, moderate recreational activities, blood urea nitrogen, creatinine, uric acid, eGFR, and urine creatinine
Ye et al. (2023) (36)	United States	Cross-sectional study	High	9,056	Urine	OR	1.51 (1.10, 2.06)	Sex, age, ethnicity, education, alcohol consumption, smoking, family income/poverty, diabetes, BMI, physical activity, cardiovascular disease, and urinary creatinine
Wang et al. (2023) (38)	United States	Cross-sectional study	Moderate	1,244	Urine	OR	1.87 (0.80, 4.34)	–
Zhao et al. (2023) (24)	United States	Cross-sectional study	Moderate	7,809	Urine	OR	1.85 (1.35, 2.53)	Sex, age, ethnicity, education, household poverty-to-income ratio, marital status, serum cotinine, BMI, urinary creatinine, vitamin C, kidney failure, gout, cancer, activity
Li et al. (2022) (32)	China	Case-control	High	740	Blood	OR	1.61 (1.1, 2.34)	Age, sex, BMI, nationality, marital status, occupation, education level, systolic blood pressure, diastolic blood pressure, smoking status, and drinking status, creatinine, urea, and uric acid
Liu et al. (2022) (25)	China	Cross-sectional study	Moderate	5,792	Urine	OR	0.52 (0.36, 0.75)	Age, BMI, systolic blood pressure, diastolic blood pressure, serum creatinine, glomerular filtration rate, serum urea, uric acid, urine protein, smoking status, and alcohol intake
Huang et al. (2021) (29)	China	Case-control	Moderate	1,572	Urine	OR	Male: 1.41 (0.67, 2.98), Female: 1.69 (0.97, 2.93)	Length of residency, BMI, nationality, family income, education level, smoking, alcohol drinking, and hypertension
Sun et al. (2019) (37)	United States	Cross-sectional study	High	29,199	Urine, blood	OR	Blood*: 1.36 (0.98, 1.87), Urine*: 2.37 (1.12, 5.04)	Age, sex, ethnicity, body mass index, socioeconomic characteristics (including educational level, marital status, and annual family income), smoking, physical activity, total energy intake, and intakes of calcium, phosphate, sodium, potassium, magnesium, total fluid, alcohol, caffeine, vitamins B6, C, and D, and estimated glomerular filtration rate
Hara et al. (2016) (30)	Belgium	Cross-sectional study	High	1,302	Urine, blood	HR	Blood*: 1.13 (0.93, 1.38), Urine*: 1.23 (0.98, 1.54)	Sex, age, serum magnesium, and 24-h urinary volume and calcium
Kaewnate et al. (2012) (19)	Thailand	Case-control	High	1,085	Urine	OR	2.73 (1.16, 6.42)	Sex, age, smoking status, and alcohol consumption
Swaddiwudhipong et al. (2011) (26)	Thailand	Cross-sectional study	Moderate	6,748	Urine	OR	Male: 1.093 (1.051, 1.138), Female: 1.039 (0.995, 1.084)	Age, alcohol consumption, body mass index, diabetes, hypertension, and urinary cadmium
Ferraro et al. (2011) (28)	United States	Cross-sectional study	Moderate	15,690	Urine	OR	1.40 (1.06, 1.86)	Age, ethnicity, body mass index, smoking, region of residence, and daily intake of calcium and sodium

(Continued)

TABLE 1 (Continued)

Author (year)	Country	Study design	Study quality	Sample size	Biological sample type	Measure of effect	RR (kidney stone risk) (95% CI)	Adjustment factors
Swaddiwudhipong et al. (2010) (33)	Thailand	Cross-sectional study	Moderate	795	Urine	OR	0.99 (0.94, 1.03)	Age, sex, smoking, body mass index, urinary cadmium, diabetes, urinary stone, hypertension, and urinary calcium
Järup et al. (1997) (31)	Sweden	Cross-sectional study	Moderate	46	Blood	OR	5.6 (1.03, 30.2)	-
Järup et al. (1993) (34)	Sweden	Cross-sectional study	Moderate	765	Urine, blood	IRR	Blood*: 3.2 (1.3, 8.3) Urine*: 1.6 (0.5, 5.0)	-
Elinder et al. (1985) (27)	Sweden	Cross-sectional study	Low	58	Urine	OR	8.9 (1.01, 77.9)	-
Thomas et al. (2013) (35)	Sweden	Cohort	Moderate	68,595	Dietary	HR	Male: 0.97 (0.77, 1.23), Female: 0.99 (0.89, 1.43)	BMI, overweight, obesity, alcohol consumption, cigarette use, and dietary intake of calcium, iron, magnesium, potassium, vitamin B6, and vitamin C

*Blood cadmium levels and kidney stone risk; *Urinary cadmium levels and kidney stone risk; OR, Odds ratio; RR, Relative risk; HR, Hazard ratio; IRR, Incidence rate ratios; BMI, Body mass index; eGFR, Estimated glomerular filtration rate.

evaluated studies ($I^2 = 76.4\%$, $p < 0.001$). Table 2 shows the results of the subgroup analyses performed to evaluate any potential effects of sex, study design, region, and exposure assessment method on these associations.

In addition, 14 studies involving 89,630 individuals in total evaluated the association between urinary cadmium exposure levels and the risk of kidney stones. The results showed that urinary cadmium exposure was associated with an increased risk of kidney stones (RR = 1.19; 95% CI: 1.08–1.30; $p < 0.001$; Table 2). A high degree of heterogeneity was observed among the evaluated studies ($I^2 = 80.00\%$, $p < 0.001$). The results of the subgroup analyses based on sex showed that cadmium exposure was associated with an increased risk of kidney stones in mixed-sex populations (RR = 1.66; 95% CI: 1.27–2.18, $I^2 = 84.70\%$, $p < 0.001$), compared to women (RR = 1.36; 95% CI: 0.94 to 1.98, $I^2 = 71.90\%$, $p = 0.002$) and men (RR = 1.01; 95% CI: 0.77–1.33, $I^2 = 83.50\%$, $p < 0.001$) (Table 2). Moreover, a statistically significant increased association was observed in cadmium-contaminated areas (RR = 1.08; 95% CI: 1.00–1.15, $I^2 = 67.8\%$, $p = 0.002$), but no association between higher cadmium exposure and the risk of kidney stones was observed in non-contaminated areas (RR = 1.33; 95% CI: 0.97–1.82, $I^2 = 83.90\%$, $p < 0.001$) (Table 2). The subgroup analyses based on study design showed that cadmium exposure was associated with an increased risk of kidney stones in both case–control studies (RR = 1.78; 95% CI: 1.20–2.64, $I^2 = 0.00\%$, $p = 0.504$) and cross-section studies (RR = 1.16; 95% CI: 1.06–1.27, $I^2 = 81.90\%$, $p < 0.001$) (Table 2). The results of the subgroup analyses by region showed that cadmium exposure was associated with an increased risk of kidney stones in non-Asian populations (RR = 1.47; 95% CI: 1.21–1.77, $I^2 = 57.10\%$, $p < 0.013$), whereas no significant association was observed in Asian populations (RR = 1.03; 95% CI: 0.94–1.13, $I^2 = 58\%$, $p = 0.015$) (Table 2). Furthermore, based on the three studies included in the linear dose–response meta-analysis, a significant association was observed between urinary cadmium exposure levels and the risk of kidney stones. Each additional 1 µg/L increase in urinary cadmium was associated with a 7% higher risk of kidney stones (RR = 1.07; 95% CI 1.01–1.13; $I^2 = 26.4\%$; three studies; 1,769 cases; range of cadmium level = 0.08–4.225ug/L; Figure 3).

A total of five studies involving 4,425 individuals in total evaluated the association between blood cadmium exposure levels and the risk of kidney stones. A significant association was found between blood cadmium exposure levels and the risk of kidney stones (RR = 1.49; 95%CI: 1.10–2.02, $I^2 = 59\%$, $p = 0.045$, Table 2) The results of the subgroup analyses by sex showed that cadmium exposure was associated with an increased risk of kidney stone disease in female individuals (RR = 1.57; 95% CI: 1.05–2.34) and mixed-sex populations (RR = 1.49; 95% CI: 1.10–2.02, $I^2 = 59\%$, $p = 0.045$) compared to male individuals (RR = 1.23; 95% CI: 0.77–1.97) (Table 2). In the subgroup analyses using a random effects model, there were significant associations between subgroups based on study design and region (Table 2). Moreover, there was a statistically significant increased risk in cadmium-contaminated areas (RR = 1.69; 95% CI: 1.05–2.71, $I^2 = 68.9\%$, $p = 0.022$), while no association was observed between higher cadmium exposure and the risk of kidney stones in non-contaminated areas (RR = 1.36; 95% CI: 0.98–1.88) (Table 2).

One cohort study examined the association between higher cadmium exposure in dietary sources and the risk of kidney stones,

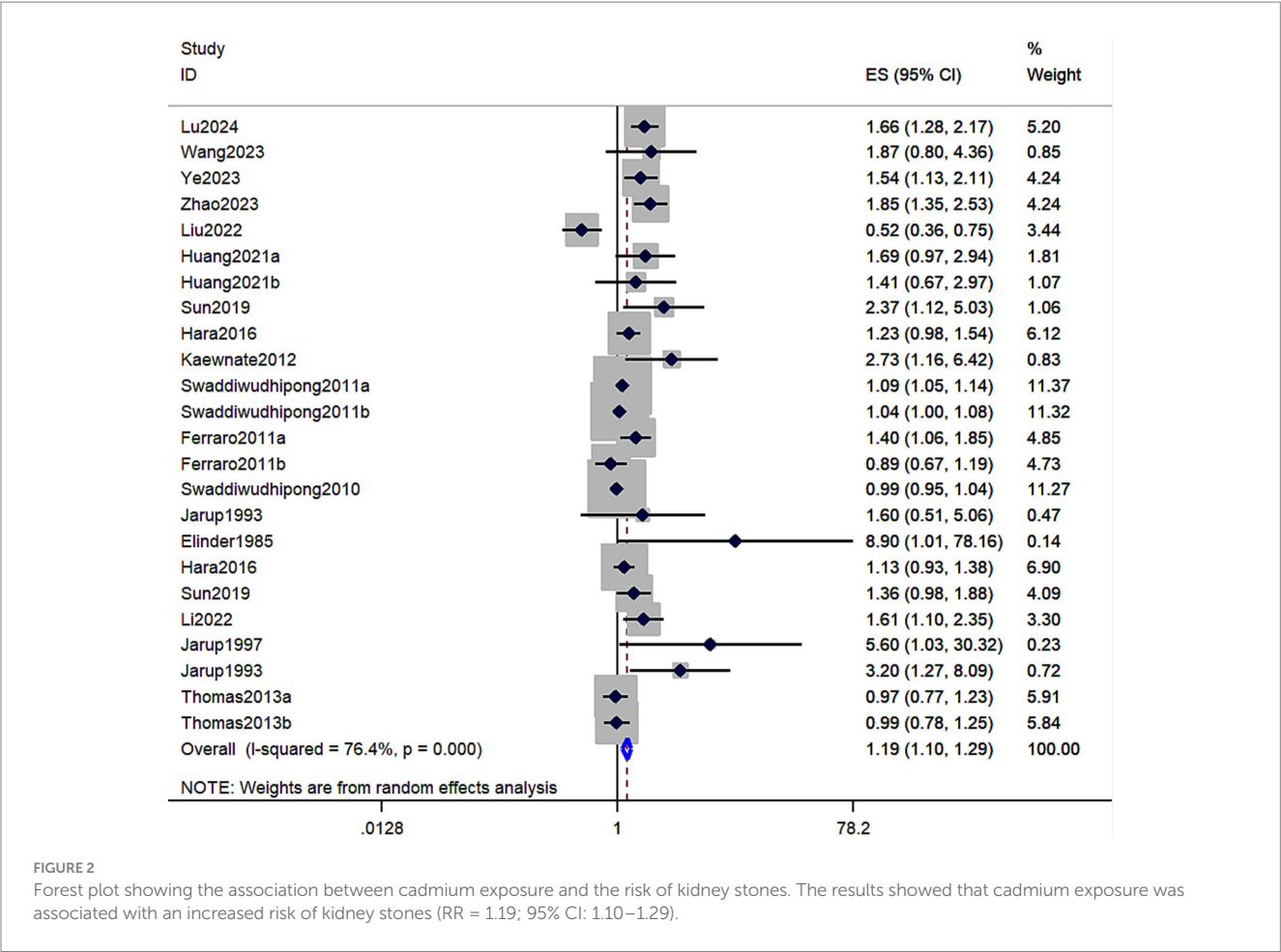


FIGURE 2 Forest plot showing the association between cadmium exposure and the risk of kidney stones. The results showed that cadmium exposure was associated with an increased risk of kidney stones (RR = 1.19; 95% CI: 1.10–1.29).

and the results showed no significant association (RR = 0.98; 95% CI: 0.83–1.16) (Table 2).

Sensitivity analysis and publication bias

A sensitivity analysis was conducted to assess the risk of kidney stones by excluding individual studies one at a time, and the results showed that no individual study influenced the overall RRs (Figure 4), indicating that the results of this meta-analysis are relatively stable. Publication bias was observed in the results based on the Egger's test and funnel plots (Table 3; Figure 5).

Discussion

A total of 17 studies involving 159,011 participants met the inclusion criteria and were eventually included in our systematic review and meta-analysis. Overall, our results indicated that the risk of kidney stones was increased in individuals with higher cadmium exposure. Importantly, we observed an increased risk of kidney stones in individuals living in cadmium-contaminated areas, while no such risk was found in non-contaminated areas. Moreover, the dose-response meta-analysis indicated that an increase of 1ug/L in urinary cadmium was associated with a 7% rise in the risk of developing kidney stones. These findings are of great significance,

as they highlight the association between cadmium exposure and the risk of kidney stones and may help in preventing the formation of kidney stones by minimizing exposure to cadmium, especially in cadmium-contaminated areas.

The heavy metal cadmium is one of the most toxic industrial and environmental pollutants, which poses a severe threat to human health (10, 11). Cadmium can enter the body through air, water, soil, and food, and it largely accumulates in the kidneys, liver, bones, and other organs, causing irreversible damage to the target organs (18, 40). Higher cadmium exposure has also been considered a possible risk factor for kidney stones. Similar to our findings, higher cadmium exposure has previously been associated with the risk of kidney stones in several studies utilizing data from the National Health and Nutrition Examination Survey (NHANES) (24, 36, 37). Kaewnate et al. also observed an association between elevated levels of urinary cadmium and urinary stones among 1,085 study residents from 13 cadmium-contaminated villages in Thailand (19). In their results, elevated levels of urinary cadmium appeared to increase the risk of urinary stones, with an adjusted odds ratio (OR) of 2.73 and a 95% confidence interval (CI) of 1.16–6.42, after adjusting for other co-variables (19). A case-control study conducted by Li et al. showed that the ratio of plasma cadmium to kidney stones in the highest quartile was 1.606 (95% CI, 1.100–2.344) compared to the lowest quartile in rural areas of Guangxi, China (32). However, several studies found no association between higher cadmium exposure and the risk of kidney stones.

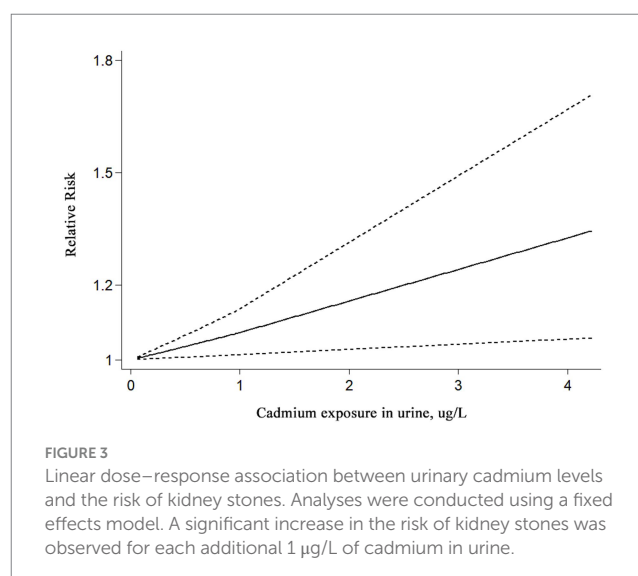
TABLE 2 Subgroup analyses of the studies included in the meta-analysis.

Subgroup	Number of studies	Pooled RR (95% CI)	I^2 statistics (%)	P -value for the heterogeneity Q test
Urinary cadmium levels	14	1.19 (1.08, 1.30)	80.00	<0.001
Female	6	1.36 (0.94, 1.98)	71.90	0.002
Male	7	1.01 (0.77, 1.33)	83.50	<0.001
Mixed*	10	1.66 (1.27, 2.18)	84.70	<0.001
Cadmium-contaminated areas	7	1.08 (1.00, 1.15)	67.80	0.002
Non-contaminated areas	6	1.33 (0.97, 1.82)	83.90	<0.001
Asian	5	1.03 (0.94, 1.13)	81.60	<0.001
Non-Asian	9	1.47 (1.21, 1.77)	57.10	0.013
Cross-sectional studies	12	1.16 (1.06, 1.27)	81.90	<0.001
Case-control studies	2	1.78 (1.20, 2.64)	0.00	0.504
Blood cadmium levels	5	1.49 (1.10, 2.02)	59.00	0.045
Female	1	1.57 (1.05, 2.34)	–	–
Male	1	1.23 (0.77, 1.97)	–	–
Mixed*	5	1.49 (1.10, 2.02)	59.00	0.045
Cadmium-contaminated areas	4	1.69 (1.05, 2.71)	68.90	0.022
Non-contaminated areas	1	1.36 (0.98, 1.88)	–	–
Asian	1	1.61 (1.10, 2.35)	–	–
Non-Asian	4	1.51 (1.01, 2.25)	63.60	0.041
Cross-sectional studies	4	1.51 (1.01, 2.25)	63.60	0.041
Case-control studies	1	1.61 (1.10, 2.35)	–	–
Dietary levels	1	0.98 (0.83, 1.16)	–	–
Cohort	1	0.98 (0.83, 1.16)	–	–

*The study population included both male and female individuals; RR, Relative risk.

Hara et al. observed that higher levels of blood cadmium and urinary cadmium were not associated with an increased risk of kidney stones (30). Liu et al. reported that higher blood cadmium exposure was not associated with the risk of kidney stones in the Qiandongnan Prefecture, China (25). This difference between studies may be due to the study design, sample size, nationalities, or study regions. Thus, more high-quality studies are needed to further assess the associations.

A meta-analysis conducted by Guo et al., which included six studies with a total of 88,045 participants, found that higher cadmium exposure was significantly associated with an increased risk of urolithiasis, with a 1.32-fold increase in risk (41). Their study focused exclusively on cadmium levels in urine and dietary intake. Compared to the previous meta-analysis, we extended this investigation by including blood cadmium levels as an additional factor. Consequently, we included more studies with a larger sample size and performed multiple subgroup analyses to assess heterogeneity and publication bias. According to the subgroup analysis, urinary and blood cadmium levels were associated with the risk of kidney stones, while dietary cadmium exposure was not significantly associated with the risk of kidney stones. A low exposure dose and limited number of studies may explain the lack of association between dietary cadmium exposure and the risk of kidney stones. In addition, a linear dose-response meta-analysis was conducted, which showed a significant association between cadmium exposure in urine and the risk of kidney stones,



with an increased risk observed for every additional 1 $\mu\text{g/L}$ of cadmium in urine. In the subgroup meta-analyses based on study design, we found that higher cadmium exposure was associated with an increased risk of kidney stones in both case-control and cross-sectional studies. We also observed a higher risk of kidney stone disease in

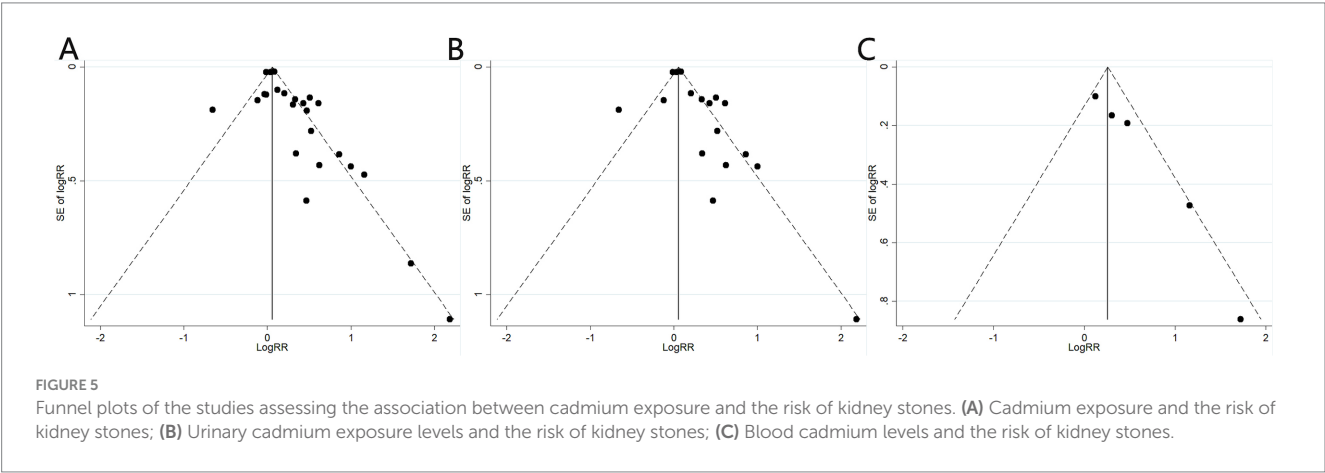
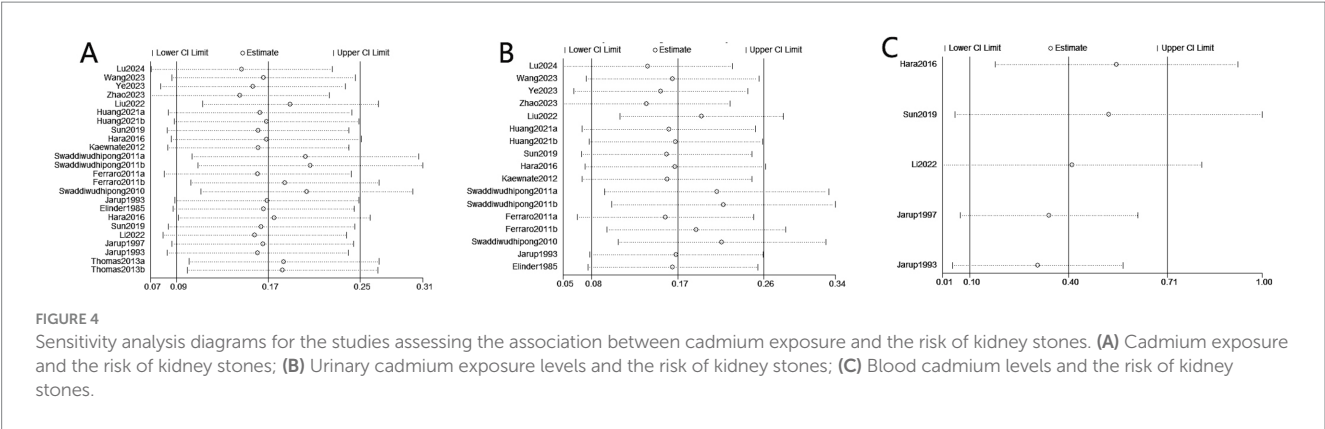


TABLE 3 Publication bias test for the association between cadmium exposure and the risk of kidney stones.

Exposure	Egger's test			Begg's test
	Coefficient	P	95% CI	
Cadmium	1.441	0.003	0.540–2.343	0.130
Urinary cadmium levels	1.459	0.025	0.208–2.709	0.773
Blood cadmium levels	2.532	0.005	1.447–3.617	0.086

individuals living in cadmium-contaminated areas but not in those from non-contaminated areas. One possible explanation for the lack of association in non-contaminated areas is that exposure levels were not high enough to significantly affect the urinary composition. The results of the subgroup analyses based on sex showed that urinary cadmium exposure was associated with an increased risk of kidney stones in mixed-sex populations compared to women and men. Similarly, blood cadmium exposure was not associated with an increased risk of kidney stones in men. The subgroup analyses based on ethnicity showed that urinary cadmium exposure was not associated with an increased risk of kidney stone disease in Asian populations compared to non-Asian populations. This lack of association is likely due to the limited number of studies included in the meta-analysis. Cadmium exposure may lead to renal stone formation, but the exact mechanism is not clear. Cadmium has a long half-life, and once it enters the body, it accumulates irreversibly in the kidneys (42, 43). Cadmium accumulation in the kidneys leads to a higher calcium excretion rate, which can raise the likelihood of developing kidney stones (12, 44).

Cadmium can recombine with metallothionein produced by renal tubular epithelial cells, causing significant damage to kidney tubular cells and impairing their reabsorption function (45, 46). Cellular injury in renal tubular epithelial cells induces crystal nucleation and aggregation, and this may result in ineffective crystallization modulators and localized areas of supersaturation in the interstitial space (47). Kidney stones may form as a result of this process. In addition, sexual hormones may play an important role in the development of nephrolithiasis (47, 48). As an endocrine-disrupting chemical, cadmium may contribute to the formation of renal stones by disrupting endocrine functions (49).

For the purpose of reporting our observations, we conducted a comprehensive literature search following the PRISMA guidelines. Our study included 17 studies involving 159,011 participants to evaluate the relationship between cadmium exposure and the risk of kidney stones. The large sample size is an important strength of this study. However, there were several limitations. First, the studies included in the meta-analysis showed a high level of heterogeneity,

which persisted even after extensive sensitivity analysis and several subgroup analyses. Second, there were too few studies to draw a definitive conclusion about the risk of kidney stones in people with dietary cadmium exposure. Finally, due to limited data, we could not assess the dose–response relationship between blood cadmium levels and the risk of kidney stones. Similarly, we were unable to assess the effects of age, smoking habits, ethnicity, and study quality on the risk of kidney stones associated with cadmium exposure. Therefore, more prospective cohort studies that evaluate the incidence of kidney stones in relation to cadmium exposure are needed.

The results of this meta-analysis strengthen the evidence that higher cadmium exposure is a risk factor for kidney stones. Further detailed research is needed to better understand the mechanisms underlying these associations. Efforts to reduce cadmium exposure in the population may help reduce the individual, economic, and societal burdens of kidney stones.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

Z-JR: Conceptualization, Methodology, Writing – original draft. QZ: Data curation, Writing – original draft. N-XT: Data curation, Software, Writing – original draft. Y-DL: Data curation, Investigation, Writing – review & editing. D-LL: Writing – review & editing. A-LL: Methodology, Software, Writing – review & editing.

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CY: Data curation, Software, Writing – review & editing. FW: Supervision, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This work was supported by Postdoctoral Science Foundation of Chongqing; Grant ID: CSTB2022NSCQ-BHX0675.

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