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# Cuproptosis contributes to Tripterygium glycosides-induced testicle injury in male mice

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# Cuproptosis contributes to Tripterygium glycosides-induced testicle injury in male mice

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## Abstract:

*Background:* Tripterygium glycosides (TG) are saponin active ingredient extracted from the peeled root of Tripterygium wilfordii Hook F. Potential toxic side effects of TG may lead to testicle injury, with the molecular mechanisms underlying this toxicity remaining insufficiently elucidated. Cuproptosis is a recently discovered mechanism of programmed cell death, which is also the focus of this research. This study aims to explore the impact of Tripterygium glycosides on testicular injury and deeply analyze the mechanisms behind it, paying special attention to the processes of cuproptosis.

*Methods:* Forty male ICR mice were randomly divided into two groups. One group of daily oral doses of sodium carboxymethyl cellulose (CMC-Na) was used as a normal control group, and the other group was administered continuously for 28 days at a dose of 100 mg/kg/d induced testicular damage through Tripterygium glycosides. Testicular tissue samples were collected from all animals for assessments of serum biochemical levels, testicular tissue pathology, and evaluation of testicular injury. The effects of Tripterygium glycosides on testicular alterations were analyzed by HE staining, while hormonal levels were determined by ELISA kits. In addition, q-PCR and Western blotting techniques were used to analyze the expression of cuproptosis-related genes and proteins in testicular tissue.

*Results:* The examination results showed that mice with testicular damage due to the intake of Tripterygium glycosides compounds had decreased weight, diminished size of the testes, as well as histopathological harm. Significant upregulation of testicular tissue cell apoptosis, along with increased levels of Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and Testosterone (T) were observed, further indicating testicle injury. The gene expressions of cuproptosis-related markers in testicular tissue, including Ferredoxin-1 (FDX1), Dihydrolipoamide-S-acetyltransferase (DLAT), Glutaminase (GLS), Lipoic acid synthetase (LIAS), Pyruvate Dehydrogenase E1 Beta Subunit (PDHB), Pyruvate Dehydrogenase E1 Subunit Alpha 1 (PDHA1), Cyclin dependent kinase

inhibitor 2A Gene (CDKN2A), Lipoyltransferase 1 (LIPT1) and ATPase Copper Transporting Beta (ATP7B) were found to be downregulated by Tripterygium glycosides in mice. Conversely, the expressions of Dihydrolipoamide Dehydrogenase (DLD), Metal Regulatory Transcription Factor 1 (MTF-1) and Solute Carrier Family 31 Member 1 (SLC31A1) were found to be upregulated in response to testicle injury induced by Tripterygium glycosides in mice. Additionally, Tripterygium glycosides was observed to downregulate the protein expressions of PDHB, GLS, DLAT, MTF-1 and LIAS.
*Conclusion:* Continuous administration of Tripterygium glycosides induces testicular injury in male mice, partly involving in the cuproptosis signaling pathway.

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# 1 INTRODUCTION

Fat-soluble active compounds derived from the roots of plants in the Celastraceae family, recognized as T. wilfordii polysaccharides or Multiglycosides of Tripterygium wilfordii (GTW), are substances of interest[1]. Clinically, these compounds are commonly used to treat conditions such as rheumatoid arthritis and nephrotic syndrome, among others. Tripterygium glycosides (TG) is a commonly utilized non-steroidal immunosuppressant in clinical practice due to their diverse therapeutic properties, including anti-inflammatory, anti-tumor, and immunosuppressive effects[2,3]. TG is commonly used to treat rheumatoid arthritis and various other autoimmune and inflammatory diseases[4-6]. Despite its beneficial anti-inflammatory and immunosuppressive properties, accumulating studies have identified that the complex chemical composition of TG can cause serious toxicity to the reproductive system, and common side effects include nausea, decreased appetite, thrombocytopenia and decreased levels of white blood cells and sperm have been reported[7].

TG may led to spermatogenic tubule atrophy, epithelial cell damage in spermatozoa and reduce sperm production[8], with these effects intensifying over time. Consequently, the narrow therapeutic window and susceptibility to testicle injury significantly constraint the clinical utility of TG[6].

Copper (Cu) is vital for all living organisms, playing a crucial role in various biological processes. It significantly influences signaling pathway by regulating or initiating biological

responses to external stimuli[9], including mitochondrial respiration, iron absorption, and antioxidant/detoxification mechanisms[10]. Copper accumulation is also associated with proliferation, growth, angiogenesis and metastasis[11]. Maintaining stable copper levels is essential for numerous physiological functions, as abnormal copper bioavailability in cells can induce oxidative stress and cytotoxicity[12]. Copper-induced cell death is a distinct pathway that relies on copper accumulation within cells, leading to impaired mitochondrial respiration and apoptosis. TG-induced spermatogonial and testicular dysfunctions in male rats are widely recognized[13,14], with reproductive toxicity linked to inflammation, oxidative stress and ferroptosis[15]. Nevertheless, the potential role of cuproptosis in the pathogenesis of testicle injury induced by TG remains to be elucidated.

Based on the aforementioned information[6], it is crucial to investigate the mechanism of testicular injury induced by TG and its impact on male reproductive function, potentially leading to infertility. In the present study, to gain insights into the toxicity mechanisms of TG, thorough analyses of testicular samples from TG-exposed mice were conducted and identified whether regulation of the cuproptosis pathway contributes to testicle injury. These examinations aim to enhance the understanding of TG's mechanisms and provide a reference for its clinical use and development.

# 2 MATERIALS AND METHODS

## 2.1 Chemicals and Reagents

Tripterygium glycoside tablets were obtained from Zhejiang Deende Pharmaceutical Company (Zhejiang, China). Luteinizing Hormone (LH), Testosterone (T) and Follicle Stimulating Hormone (FSH) assay kits (cat. E-EL-M3053, cat. E-EL-M0003 and cat. E-EL-M0511), along with the One-step TUNEL In Situ Apoptosis Kit (Green, FITC) assay kits (cat. E-CK-A320), were purchased from Elabscience Biotechnology Co., Ltd. The ChamQ Bule Universal SYBR qPCR Master Mix (Catalog No. Q312-03) was purchased from Nanjing Vazyme, The Western and IP Cell Lysate buffer (cat. no. BL509A) and 100 mM phenylmethylsulfonyl fluoride (PMSF, cat. no. BL507A-1) were obtained from Biosharp (Beijing, China). The ProtLytic Protease and Phosphatase Inhibitor Cocktail (cat. no. P002) was obtained from New Cell & Molecular Biotech Co., Ltd, the BCA protein assay kit (cat. no. P0012S) was procured from Beyotime Institute of Biotechnology, located in Shanghai,

China. Lipoate synthase protein (LIAS, cat. no. 11577-1-AP), Dihydrolipoamide S-Acetyltransferase (DLAT, cat. no. 13426-1-AP), KGA/GAC protein (GLS, cat. no. 13426-1-AP), Metal Regulatory Transcription Factor 1(MTF-1, cat. no. 25383-1-AP), Pyruvate Dehydrogenase E1 Subunit Beta (PDHB, cat. no. 13426-1-AP) primary antibodies and Anti-Rabbit IgG (cat. no. SA00001-2) or Anti-Mouse IgG (cat. no. RGAM001) were obtained from ProteinTech Group. Anti-β-actin antibodies was obtained from Sigma-Aldrich (cat. no. A1978), while other chemical reagents were procured from Beyotime Biotechnology in Shanghai, China.

## 2.2 Animals and experimental design

Forty male ICR mice (body weight between 22 and 25 g), of specifc pathogen-free (SPF) grade, were introduced one week before the experiment. They were provided by the Department of Vital River Company (Beijing Vital River Laboratory Animal Technology Co., Ltd.). The animal experiments adhered to the experimental guidelines and globally accepted standards for animal care, as approved by the Laboratory Animal Ethics Committee of Zhejiang Pharmaceutical University (Ningbo, China) . Food and water were provided ad libitum, and bedding in the cages was changed every other day to keep cleanliness and dryness. All animals have adapted to this controlled laboratory conditions, with 60 ± 10% humidity, temperatures maintained at 24±2°C, and a 12-hour light-dark cycle (lights on at 8:00 a.m.).

A total of 40 male mice were randomly divided into two groups, followed by 5 to 7 days of adaptation. Among them, the mice in the normal group were treatment with a 0.5% solution of sodium carboxymethyl cellulose (CMC-Na, Shandong Yousuo Chemical Co., LTD, FH9), while the mice in the TG group were received TG via gavage once daily for 4 weeks at a dose of 100 mg/kg/d. The choice of this dose was based on previous studies aimed at establishing testicular injury models and does not directly correspond to the human clinical dose. All medications were administered by gavage. During the experiment, the researchers monitored the weight changes and food intake of these mice every day. After 28 days, all mice were euthanized at the end of the experimental period via cervical dislocation under deep anesthesia induced by intraperitoneal injection of sodium pentobarbital at a dose of 150 mg/kg body weight. Anesthesia was confirmed by the absence of reflexes (e.g., toe pinch and corneal reflex) prior to cervical dislocation. Blood was collected by eyeball extirpation, and testicular tissues were excised from all mice following a 12-hour fasting

period. One testicular tissue was fixed in 4% paraformaldehyde Fixative, and the other was stored at -80°C for further analysis.

## 2.3 Weight changes and testis index

Mice were subjected to regular body weight measurements every three days to monitor track weight fluctuations over time. Following the conclusion of the gavage procedure, the researchers recorded the final body weights of these mice. Additionally, the weight of the testis was also in order to calculate the organ index according to the following formula: relative organ weight (%) = (wet weight of organ [g] / body weight [g]) × 100 %.

## 2.4 Blood biochemical analysis

The blood samples were for 15 minutes at a speed of 7,500 rps/min. LH, T and FSH levels were assessed utilizing mouse-specific ELISA kits and the operation is strictly in accordance with the manufacturer's instructions. The sensitivity of the kit was defined as a concentration that could be reliably differentiated from zero, exhibiting a coefficient of variation (CV%) of less than 15%. The T ELISA kit employs the Competitive-ELISA principle, while the Luteinizing Hormone and Follicle Stimulating Hormone ELISA kits used the Sandwich-ELISA principle. Finally, the Varioskan LUX Multifunctional microplate was used to quantitatively determine the concentrations of LH, T and FSH in serum at a wavelength of 450 nm.

## 2.5 Histopathological analysis

The testes were stored in 4% paraformaldehyde at least 24 hours for subsequent histopathological examinations. After fixation, the specimens need to be dehydrated through a series of ethanol solutions in turn, then embedded in paraffin wax and sectioned into 4 μm thick slices. Paraffin was removed from the sections with xylene, followed by tissue rehydrated through an ethanol gradient. The sections were then stained with HE for morphological assessment. The morphological structure of the testes was examined using the Japanese Olympus CX23 microscope.

## 2.6 TUNEL Assay

Testicular tissue samples were evaluated using the TUNEL method with an In Situ Cell Death Detection kit according to the operating guidelines provided by the manufacturer. The paraffin-embedded tissue samples were deparaffinized and hydrated using a conventional protocol: deparaffinization was performed twice with xylene for 10 minutes each, followed by immersion in anhydrous alcohol for 5 minutes, and then through three consecutive concentrations of 90%, 80%

and 70% ethanol aqueous solutions for 3 minutes each. The samples after dewaxing are put into the PBS buffer, soaked for 5 minutes each time, and washed three times in total. After that, each sample was then treated with 50 μL of 1× protease K and incubated at room temperature for 20 minutes. The adequately permeated samples were rinsed three times in PBS for 5 minutes each time.

Next, 100 μL of Equilibration solution (1×) was dispensed into each sample and allowed to equilibrate at room temperature for 15-30 minutes. After removing the equilibration solution with suction paper, 50 μL of TdT enzyme working solution was added to each sample. Then, the samples were covered with coverslips and put in a humid chamber at 37°C in the dark for 60 minutes. Following this incubation, the samples were rinsed three times in PBS, with each rinse lasting 5 minutes. Excess water was blotted dry, and DAPI was added and incubated at room temperature in darkness for 5 minutes to counterstain the nuclei. The samples were then submerged in PBS and rinsed four times for 5 minutes each. Then, any remaining liquid was removed by blotting with absorbent paper, and the samples were sealed with a tablet sealant containing an anti-fluorescence quenching agent. For microscopic analysis, an excitation wavelength wavelength of 450-500 nm and an emission wavelength range of 515-565 nm (green fluorescence) were used. The specimens were ultimately observed under a fluorescence microscope.

## 2.7 Quantitative real-time PCR Analysis

Total RNA was extracted from testicular tissues using a Trizol kit (Thermo Fisher Scientific) according to the manufacturer's instructions. cDNA was synthesized using a cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was conducted utilizing a qPCR Master Mix. The conditions for the PCR thermal cycle reaction include: first, denature treatment at 95°C for 3 minutes, followed by 30 to 35 cycles, denature at 95°C for 15 seconds in each cycle, and then annealing at 60°C for 15 seconds; finally, concluding with a final extension step at 72°C for both 15 seconds and then for an additional 60 seconds. The amplification and detection process is carried out in the MicroAmp optical 96-well reaction plate, and utilizing optical adhesive covers for enhanced integrity. Quantification of target gene expression levels was performed using the $2^{-\Delta\Delta Ct}$ method. The specific primers used for amplification of the target genes are listed in Table 1.

## 2.8 Western Blot Analysis

Proteins were isolated from testicular tissue samples using a protein extraction kit from Thermo,

and their concentrations were quantified using a Protein Assay Kit according to the manufacturer's instructions. Testicular tissue samples were lysed on ice for 15-30 minutes. At 4°C, after 15 minutes of centrifugation with a centrifugal force of 10,000 × g, the supernatant is transferred to a new test tube, and then the protein concentration is determined with a BCA assay kit.

A total of 20 μl of the protein sample and protein Marker were applied onto a 10% SDS-PAGE and subjected to electrophoresis. Following electrophoresis, the proteins were transferred to 0.22 μm polyvinylidene fluoride membranes. The membranes were then incubated in tris-buffered saline (TBST) with 5% skimmed milk for 1.5-2 hours. Then, membranes were transferred to TBST with primary antibodies respectively and incubated over night at 4°C, primary antibodies specific to DLAT, LIAS, PDHB, GLS, MTF-1, and other targets. Following three washes with TBST, the membranes were incubated with either Anti-Rabbit IgG or Anti-Mouse IgG at room temperature for a duration of 1 to 2 hours. β-actin was used as an internal normal. Immunodetection was carried out by utilizing a chemiluminescence substrate (Tanon 4200, Shanghai, China).

### 2.9 Statistical Analysis

All statistical analyses were performed with GraphPad version 9.0.2 (GraphPad Software). Data were expressed as mean ± Structural Equation Model (SEM). Comparisons among all groups were performed with t-test. A p-values of less than 0.05 was considered statistically significant.

# 3 RESULTS

### 3.1 General Indexes

Both groups of mice exhibited substantial fluctuations in body mass over the 4-week period. The primary symptoms observed in the TG group included atony, lethargy and eye secretions. Compared to the Normal group, the TG group showed a significant reduction in testicular weight by the end of the fourth week (Figure 1, 2). As illustrated in the Table 2, mice in the TG group experienced a significant decrease in the organ coefficient of the testis compared to the Normal group.

### 3.2 Biochemical Indexes

Figure 3 shows that compared with the Normal group, the serum LH level of patients in the TG group increased significantly. Additionally, TG administration led to a statistically significant elevation in serum FSH and T levels.

### 3.3 Pathological Indexes

HE staining revealed that seminiferous tubules in the Normal group had a normal round or ovoid shape with intact morphology (Figure 4). The tubular walls were complete, the spermatogenesis cells are arranged in a regular, tight and orderly manner, and mature sperm can also be seen in the lumen. The tubule walls were intact and thick, with neat borders and the distribution ratio of various types of cells is also within the normal range.

In contrast, the testicular tissue of the TG group exhibited significant histopathological alterations. These changes included atrophy of certain seminiferous tubules and the number of spermatogenic cells at all levels also decreased. High-magnification microscopy revealed a wavy and disrupted basal layer structure, with a disorganized arrangement of spermatogenic cells.

### 3.4 TUNEL Analysis

To examine whether the cuproptosis-related pathway induced by TG affects cell apoptosis, a TUNEL assay was performed. The results indicated that: Compared to the Normal group, the TG group mice exhibited a higher proportion of apoptotic cells in the testicular tissue, as indicated by TUNEL staining (Figure 5). A statistically significant difference was observed between the Normal and TG groups.

### 3.5 Cuproptosis–related gene expressions

To investigate whether the cuproptosis-related pathway played a role in TG-induced testicular injury, the expressions of Ferredoxin 1 (FDX1), Lipoyltransferase 1 (LIPT1), Cyclin dependent kinase inhibitor 2A (CDKN2A), Pyruvate dehydrogenase E1 subunit alpha 1 (PDHA1), Solute carrier family 31 member 1 (SLC31A1), ATPase copper transporting beta (ATP7B), LIAS, DLAT, GLS, PDHB and MTF-1 mRNA to investigate whether cuproptosis-related pathway played an important role in the TG-induced testicle injury. As result shown in Figure 6-A, the mRNA expressions of FDX1, LIPT1, LIAS, DLAT, GLS, PDHB, CDKN2A, MTF-1, PDHA1, SLC31A1 and ATP7B were substantially lower in the TG group compared to the Normal group. Conversely, the expressions of DLD, MTF-1 and SLC31A1 were markedly elevated increased in the TG group.

### 3.6 Cuproptosis–related protein expressions

A number of pivotal transcriptional markers of cuproptosis, such as PDHB, GLS, DLAT, MTF-1 and LIAS were assessed. As depicted in Figure 6-B, in alignment with the gene expression

findings, the protein expressions of PDHB, GLS, DLAT, MTF-1 and LIAS significantly decreased in the TG group compared to the Normal group (Figure 6-B).

# DISCUSSION

TG is the most widely used and reliable immunosuppressive Chinese patent medicine, and has exactly anti-inflammatory and kidney protective effects[16]. It is worth noting that testicular injury is a frequently observed side effect of TG, manifesting clinically as oligospermia, azoospermia, diminished sperm motility and decreased libido. Despite controversial clinical use from a narrow therapeutic range, its impact on testicular health is still lacking in sufficient research. Therefore, this study concentrates on the the molecular mechanism of TG-induced reproductive toxicity, while previous studies on reproductive toxicology have primarily utilized rat models, we specifically utilized 4-week-old mice in the present experimental design. Consistent with previous studies demonstrated toxicological profiles[17], the continuous administration of TG at a dose of 100 mg/kg over a duration of 28 days was conducted.

It was found that testicular injury was induced in the model, which subsequently manifested the corresponding pathophysiology, manifested as a significant decrease in testicular weight and testis size. Additionally, TG treatment also increased proportion of apoptotic cells in the testicular tissue of experimental mice.

FSH and T together regulate the process of sperm production. T is secreted by Leydig cells under the stimulation of LH, which plays an important roles in the differentiation of peripheral tissues and the promotion of spermatogenesis[18]. Notably, in the acute systemic inflammatory reaction caused by lipopolysaccharide intraperitoneal injection, male fecundity was compromised alongside elevated serum FSH and LH levels[19]. Furthermore, when rats ingested TG at a dose of 40 mg/kg/day for 4 consecutive weeks, the serum FSH and LH concentrations of the model group were increased significantly[20].Our study reveals that TG administration induces a significant increase in FSH, T and LH levels, which suggests that TG-treatment may resulted in disturbed spermatogenic process.

The copper-dependent lipoylation process is critical to the activity of related enzymes in the tricarboxylic acid (TCA) cycle. However, excessive intracellular copper triggers cuproptosis, a

unique form of mitochondrial respiration-dependent cell death distinct from other regulated pathways[21-24]. The core of this process is the steady-state regulation of copper, which is regulated by transport proteins such as the copper importer SLC31A1 (CTR1) and exporter ATP7B[25]. In diabetic cardiomyopathy, elevated Advanced Glycation End-products (AGEs) and copper levels upregulate SLC31A1 (CTR1), resulting in excessive accumulation of copper content in cardiomyocytes[26]. Conversely, ATP7B functions as the primary exporter of cytosolic copper[27]. Literature reports show that the β-catenin/TCF4 transcription complex promotes the expression of ATP7B, which confers resistance to cuproptosis[28]. In the present study, the results indicated that TG is capable of promoting the upregulation in the expression level of SLC31A1 gene, while simultaneously leading to a reduction in the gene expression of ATP7B in TG-induced testicle injury. Cuproptosis disrupt the balance between copper transport regulators (reducing ATP7B-mediated efflux and increasing SLC31A1-dependent influx), which could be associated with TG-induced testicular injury.

Furthermore, research investigations have the impairment of testicular spermatogenic function is associated with TCA cycle[23]. Central to this process, the activity of DLAT enzyme depends on the FDX1-mediated oligomerization reaction, which is crucial to maintaining the metabolic activity of mitochondria[24], wherein FDX1 converts $Cu^{2+}$ to $Cu^{+}$, disrupting Fe-S cluster synthesis and inducing apoptosis (Fig.7). Mechanistically, FDX1-LIAS interaction drives DLAT lipoylation, creating a copper overload/lipoylation feedforward loop that disrupts metabolic homeostasis[29,30]. Furthermore, copper disrupts hepatic lipid metabolism by activating MTF-1 (a copper homeostasis regulator)[31]. Transcriptomic analyses revealed that the deletion of FDX1 function induces a series of compensatory metabolism-related genes[32], while LIAS overexpression mitigates oxidative stress, preserves mitochondrial function, and alleviates NAFLD/NASH[33]. Despite tumor downregulation, PDHB (linking glycolysis/TCA) suppresses ccRCC cell proliferation, migration and invasion[34], PDHA1 similarly exhibits paradoxical tumor-suppressive effects by inhibiting CC cell proliferation and invasion while elevating ROS upon overexpression[35]. It is worth noting that our research data demonstrate that testicular injury caused by TG downregulates FDX1, DLAT, and LIAS gene expressions were observed in testicular tissues. TG treatment significantly suppressing PDHB and PDHA1 mRNA levels while paradoxically upregulating MTF-1 transcription but inhibiting its protein expression. This divergence may related to the post-translational modification

or proteasome degradation mechanism caused by copper overload. Notably, these synergistic disturbances may ultimately lead to testicular injury through mitochondrial dysfunction and disrupted structural equilibrium of the pyruvate dehydrogenase (PDH) complex.

DLD, a multifunctional oxidoreductase[23], which could promote the necrosis, apoptosis[36]. While LIPT1 mediates lipoic acid metabolism and has been implicated in hereditary disorders[37], yet protective in non-small cell lung cancer (NSCLC)[38]. CDKN2A (encoding p16) promotes tumor proliferation and suppresses apoptosis, while GLS drives glutamine homeostasis and oncogenesis, notably in prostate cancer (PCa) where its knockdown inhibits proliferation and induces apoptosis[39,40]. In our study, TG-induced significantly downregulated the gene expressions of LIPT1, CDKN2A, and GLS while upregulating DLD, suggesting that suggesting that this alteration may be associated with apoptosis.

# CONCLUSION

This study revealed that TG could significantly increase FSH, LH, and T levels in serum. Histopathological analysis demonstrated dose-dependent spermatocyte depletion and seminiferous tubule vacuolization in TG-treated testes. Mechanistically, TG specifically altered cuproptosis-related gene or protein expression. These results not only establish empirical validation of cuproptosis as a pathophysiological regulator in testicular toxicological responses, but also provide an experimental basis for developing preventive/therapeutic strategies and clinical applications of TG.

# ETHICS STATEMENT

The animal study was reviewed and approved by the Laboratory Animal Ethics Committee of Zhejiang Pharmaceutical College (ZYLL202302002).

# DATA AVAILABILITY STATEMENT

Data available on request from the authors.

# AUTHOR CONTRIBUTIONS

Li Zhao conducted the experimental operations and contributed to the writing of the manuscript,

Xiaomin Yao and Junling Zhu assisted in both the writing and editing processes. Shu Jia and Jingyi Wu were responsible for data statistical analysis.

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We hereby disclose that during the preparation of this manuscript, AI-assisted tools (Deepseek) were used for language polishing and grammar checking to improve the readability of the text. To ensure the manuscript fully complies with the journal's publishing ethics and to enhance the originality of the writing, we have carefully reviewed and revised the text to reduce the potential AI-generated style or phrasing.

# Abbreviations

| | |
|---|---|
| FDX1 | Ferredoxin 1 |
| LIAS | Lipoic acid synthetase |
| LIPT1 | Lipoyltransferase 1 |
| DLD | Dihydrolipoamide dehydrogenase |
| DLAT | Dihydrolipoamide-S-acetyltransferase |
| PDHA1 | Pyruvate dehydrogenase E1 subunit alpha 1 |
| PDHB | Pyruvate dehydrogenase E1 subunit be |
| MTF1 | Metal regulatory transcription factor 1 |
| GLS | Glutaminase |
| CDKN2A | Cyclin dependent kinase inhibitor 2A |
| SLC31A1 | Solute carrier family 31 member 1 |
| ATP7B | ATPase copper transporting beta |

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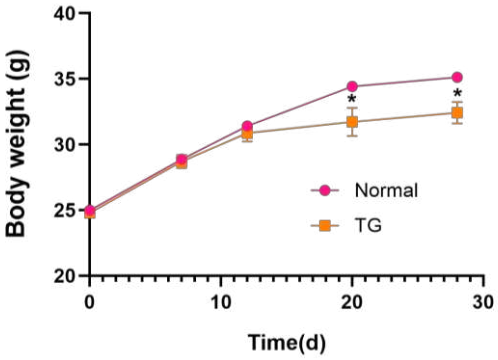



Fig. 1 TG induced obvious decrease of body weight in mice. *P<0.05 versus Normal group.

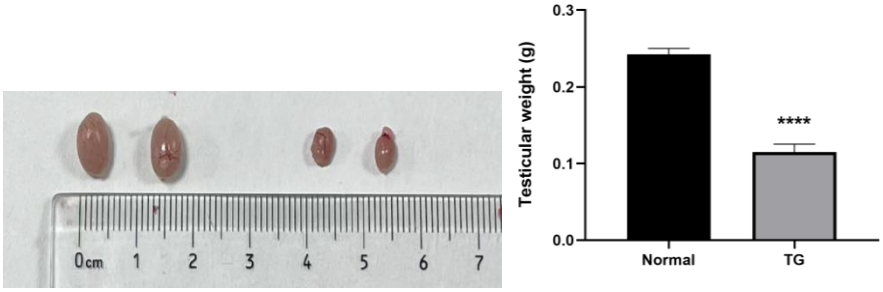



Fig. 2 TG induced significant decreases reductions in testicular weight. ****P<0.0001 versus Normal group.

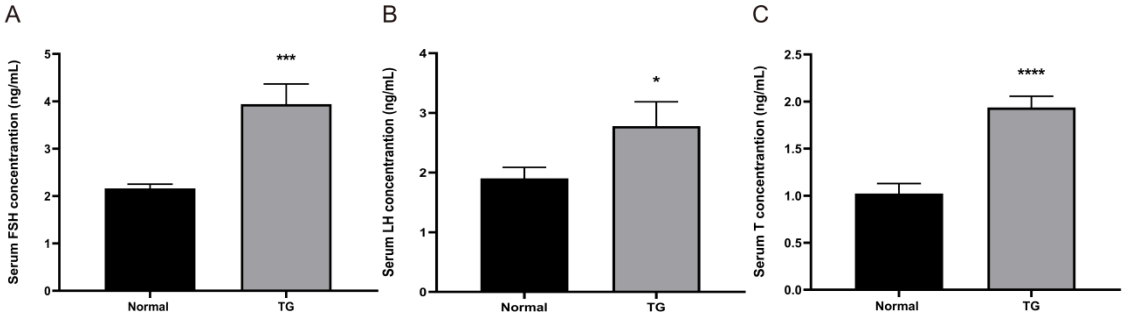



Fig. 3 TG induced the increase of serum biochemical indices in male mice (n =12). Blood samples were obtained at 4 weeks after TG administration. A, serum FSH; B, serum LH; C, serum T. *P<0.05, ***P<0.01, ****P<0.0001 versus Normal group.

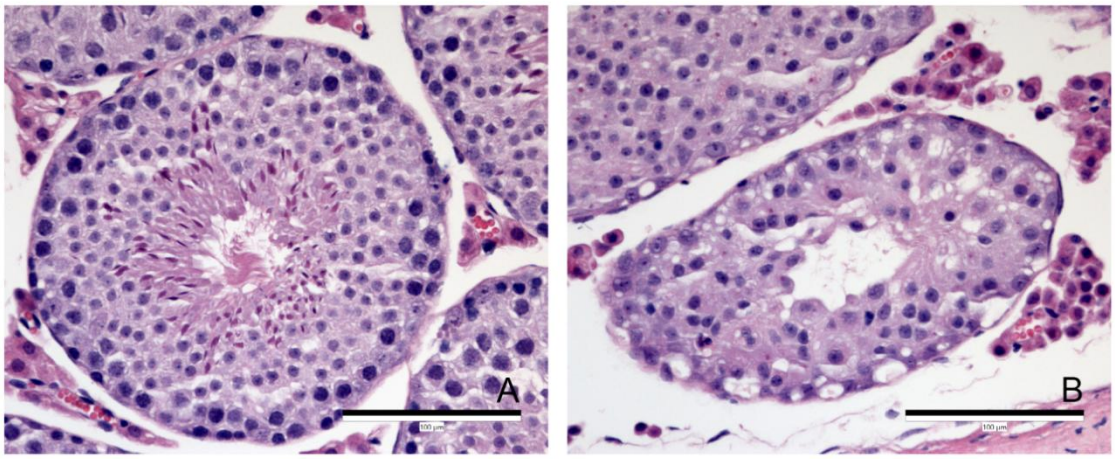


Fig. 4 TG induced the change of histopathology in male mice (n = 12). Tissue specimens were collected 4 weeks after TG administration and testis tissue sections were stained with hematoxylin-eosin. A: Testicular tissue structure in the normal group; B: Testicular tissue structure in the TG group. ( HE staining，×400).

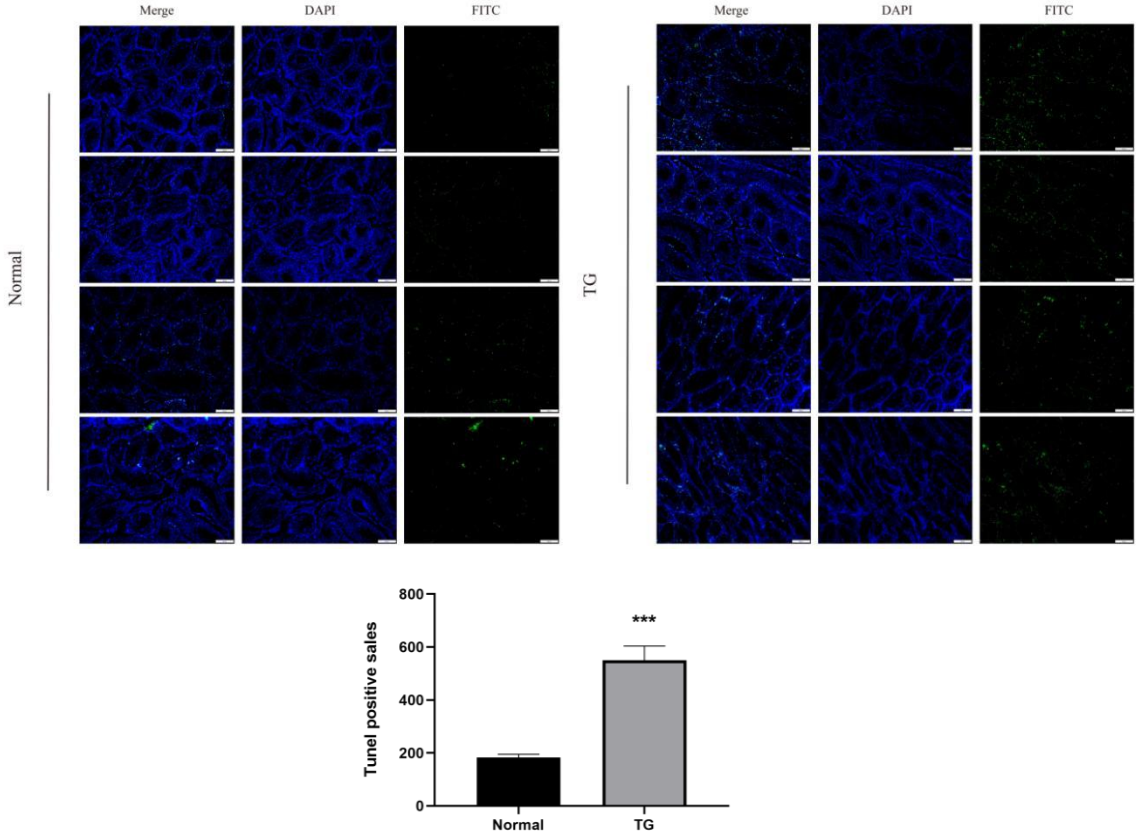



Fig. 5 TG induced cells of testis apoptosis in male mice (TUNEL assay, 200 $\mu$ m) (n = 12). Testis tissue specimens were collected at 4 weeks after TG administration. Representative TUNEL results showing apoptosis from Normal and TG group. The foci were quantified by an ImageJ software (Bethesda, MD). ***p < 0.001 versus Normal group.

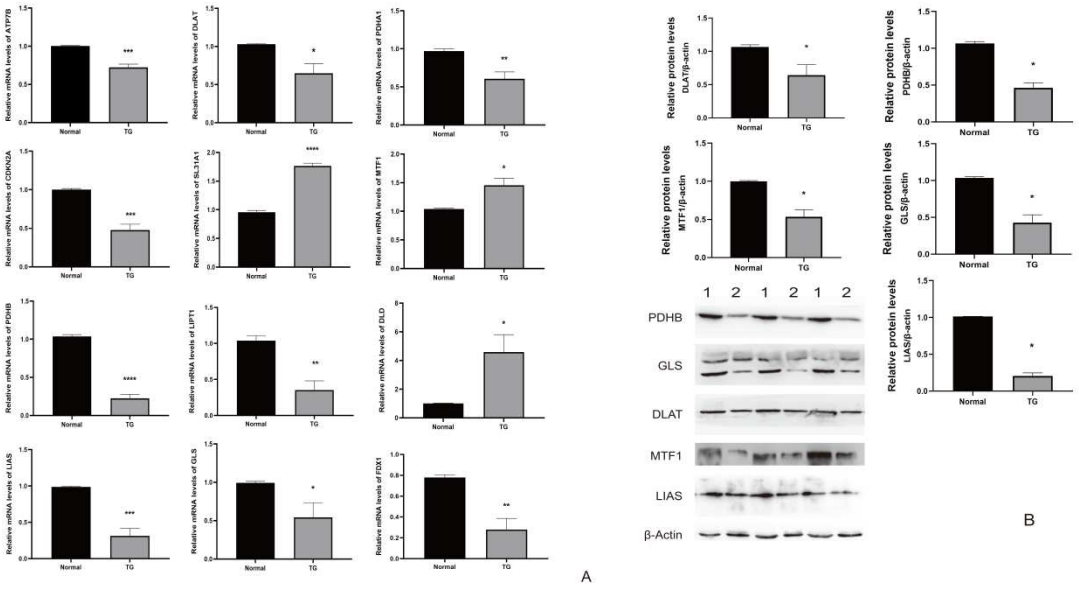


Fig. 6 The expression patterns of differentially expressed genes associated with the cuproptosis-related in male mice are shown; expression was determined by q-PCR and Western blot; All of the q-PCR data are shown as the mean ± standard error; n = 5. A: Real-time qPCR analysis showing cuproptosis marker relative mRNA expression levels of FDX1, LIAS, LIPT1, DLAT, PDHA1, PDHB, MTF1, GLS, CDKN2A, SLC31A1 and ATP7B; B: Relative protein expression levels of PDHB, GLS, DLAT, MTF1 and LIAS; Grayscale values of PDHB, GLS, DLAT, MTF1 and LIAS protein expression bands in testicle injury. Lanes 1, Normal group; lanes 2, TG group. *p < 0.05 , **p < 0.01 , ***p < 0.001 , ****p < 0.0001 versus Normal group.

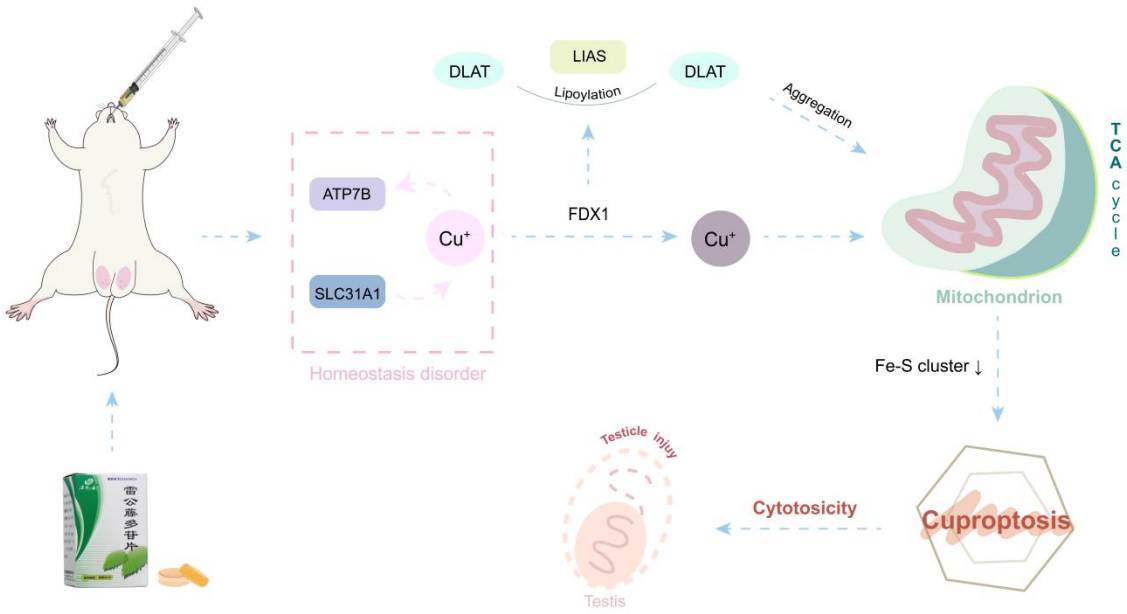



Fig. 7 Diagram illustrating a hypothetical mechanism of action for the differential expression of cuproptosis-related protein that occurred after induced with TG. FDX1: ferredoxin 1; SLC31A1: solute carrier family 31 member 1; DLAT: dihydrolipoamide acetyltransferase; LIAS: lipoate synthase; TCA: tricarboxylic acid; ATP7A/B: ATPase copper transporting alpha/beta ( figure is an original figure created by the authors of the manuscript ).

## Supplementary Files

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