

Protective effects of total flavonoids of *Astragalus membranaceus* Bunge combined with selenium-enriched yeast against zearalenone-induced reproductive injury in male mice

Wei Wang¹, Bing-jie Ge¹, Rui Sang¹, Xin-man Liu, Tian Yu, Xue-mei Zhang^{*}

College of Agriculture, Yanbian University, Gongyuan Street, Yanji, Jilin 133002, PR China

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ABSTRACT

Zearalenone (ZEA) is an environmental endocrine disruptor generated by fungi, which mainly causes reproductive injury. Total flavonoids of *Astragalus membranaceus* Bunge (TFA) is one of the most beneficial components of *Astragalus membranaceus* Bunge with a variety of pharmacological effects, selenium (Se) is a trace element with antioxidant effect, but it remains unclear whether their combination can control the reproductive toxicity caused by ZEA. This study aims to determine the effects and potential mechanisms of TFA combined with Se-enriched yeast (SeY) against ZEA-induced reproductive injury in male mice. In this study, we first utilized network pharmacology to predict the possible targets of TFA combined with SeY, and further elucidated and verified the effects and underlying mechanisms through *in vivo* experiment. Network pharmacology showed that the core targets of TFA combined with SeY against testicular damage were closely related to inflammation, oxidative stress and apoptosis. *In vivo* experiment demonstrated that TFA combined with SeY effectively improved sperm quality, alleviated pathological changes of testis, and repaired mitochondrial damage and blood-testis barrier. Moreover, TFA combined with SeY reduced ZEA-induced inflammation through suppressing the overproduction of inflammatory factors, attenuated oxidative stress through activating Keap1/Nrf2/HO-1 pathway, and mitigated apoptosis through inhibiting mitochondrial and death receptor pathways. In conclusion, TFA combined with SeY exerted protective effects against ZEA-induced testicular injury by anti-inflammatory, antioxidant and anti-apoptotic mechanisms. This offers a promising solution for controlling ZEA poisoning and provides experimental support for the combined use of traditional Chinese medicine and trace elements.

1. Introduction

Mycotoxins are secondary metabolites generated by fungi under specific environmental temperature and humidity conditions, and found in many agricultural products, which pose a huge threat to agricultural development, human and animal health (Tu et al., 2023). As one of the mycotoxins, zearalenone (ZEA), also known as F-2 toxin, is a nonsteroidal estrogen mycotoxin generated by *Fusarium* genera (Murtaza et al., 2022). ZEA can be widely detected in grains and their products. Normally, ZEA appears excellent thermal stability, and thus it is difficult to eliminate during the processing and storage of food and feed (Cai et al., 2024). At present, ZEA contamination is widespread around the world

and mainly concentrated in the northern temperate areas with low temperature and high humidity such as Europe, America and Asia (Jurascsek et al., 2022). As an estrogenic mycotoxin, ZEA can interfere with estrogen function and induce reproductive disorder, thereby exerting reproductive toxicity (Balló et al., 2023; Pang et al., 2017). The testis is one of the important reproductive organs of males, which is mainly involved in sperm production and androgen secretion (Li et al., 2024). Once the testis is damaged, it leads to a disorder of the male reproductive system, which severely hinders spermatogenesis and the normal production of sex steroids, ultimately leading to infertility (Carrageta et al., 2024).

The pathophysiology of ZEA-induced reproductive injury is complex

^{*} Corresponding author.

E-mail addresses: 1255360108@qq.com (W. Wang), 727965819@qq.com (B.-j. Ge), SR@ybu.edu.cn (R. Sang), 2326934425@qq.com (X.-m. Liu), 2644869910@qq.com (T. Yu), zhangxm@ybu.edu.cn (X.-m. Zhang).

¹ These authors made equal contributions to this research.

with various factors involved. Oxidative stress is a key factor contributing to ZEA-induced testicular damage (Yang et al., 2018). ZEA exposure leads to the overproduction of reactive oxygen species (ROS), which may further contribute to inflammation and apoptosis. The Kelch-like ECH-associated protein 1 (Keap1)/Nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway, a critical regulator of redox homeostasis, is triggered by excessive ROS (Cao et al., 2021). In addition, Studies have found that ZEA can cause apoptosis in reproductive system through the death receptor and mitochondrial apoptotic pathway (Xu et al., 2016; Wu et al., 2021). Therefore, based on the molecular processes of injury development, the main goal of ZEA-induced reproductive injury control is to reduce oxidative stress and apoptosis by modulating corresponding signal transduction pathways.

At present, physical, chemical and biological methods are the primary approaches used to remove ZEA (Zhang et al., 2023a). Physical and chemical methods have great limitations, including low detoxification efficiency, high cost, serious impact on grain flavor, damage to the absorption of nutrients in feed, and may even cause environmental pollution. Compared with the these two methods, biological method is more cost-effective and does not cause nutrient loss, and is the ideal way to solve ZEA poisoning, however, the biosafety of the microorganisms and their degradation products requires further investigation (Cai et al., 2024). In recent years, bioactive substances derived from medicinal plants and natural products have been widely used in the treatment of ZEA injury (Cai et al., 2024).

Total flavonoids of *Astragalus membranaceus* Bunge (TFA) is one of the most beneficial components of *Astragalus membranaceus* Bunge with a variety of pharmacological effects, including antioxidant, anti-inflammatory and immunomodulation (Dan et al., 2024). It has been reported that TFA protects glomerular filtration barrier in diabetic kidney disease through inhibiting inflammatory factors and attenuating oxidative stress (Liu et al., 2024). Selenium (Se) is a trace element that plays a very important role in the body with remarkable biological effects (Dong et al., 2024). Se-enriched yeast (SeY), a type of organic Se, is widely used and has been shown to be highly effective (Zhang et al., 2023). It has been reported that Se has protective effect on chronic zearalenone-induced reproductive system damage in male mice (Long et al., 2016). In addition, the combination of drugs shows superior efficacy through synergistic interaction. However, it is unclear whether the combination of TFA and SeY might have better therapeutic effects on ZEA-induced reproductive toxicity.

In the present study, we first used network pharmacology to screen targets of TFA combined with SeY against testicular injury, identify intersecting genes and perform enrichment analysis to uncover conceivable mechanisms. Subsequently, we elucidated the effects and underlying mechanisms of TFA combined with SeY on ZEA-induced reproductive injury through *in vivo* experiment. This work will offer a new idea for the prevention and treatment of ZEA poisoning, also lay a theoretical foundation for the further development of the synergistic use of traditional Chinese medicine and trace elements.

2. Materials and methods

2.1. Drugs and reagents

TFA (purity $\geq 90\%$) was obtained from Chengdu Desite Biotech Co., Ltd (Chengdu, China), mainly contained several flavonoid components: calycosin-7-glucoside, calycosin, formononetin and ononin (Yang et al., 2021). SeY (Se content of 2 g/kg) was obtained from the Angel Yeast Co., Ltd (Wuhan, China), mainly contained two organic selenium components: selenomethionine and selenocysteine. ZEA (purity $\geq 99\%$) was obtained from Pribolab Bioengineering Co., Ltd (Qingdao, China). Enzyme-linked immunosorbent assay (ELISA) kits tumor necrosis factor- α (TNF- α) (CME0004, sandwich ELISA), interleukin-1 β (IL-1 β) (CME0015, sandwich ELISA), and interleukin-6 (IL-6) (CME0006, sandwich ELISA) were purchased from Beijing Sizhengbai Biotech Co.,

Ltd (Beijing, China). Follicle-stimulating hormone (FSH) (CSB-E06871m, competitive inhibition ELISA), luteinizing hormone (LH) (CSB-E12770m, sandwich ELISA) and testosterone (CSB-E05101m, competitive inhibition ELISA) were obtained from Cusabio Biotech Co., Ltd (Wuhan, China). The terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL) (G002-2-2, fluorescence method), ROS (E004-1-1, chemical fluorescence method), malondialdehyde (MDA) (A003-1-2, thiobarbituric acid colorimetry), superoxide dismutase (SOD) (A001-1-2, hydroxylamine method), catalase (CAT) (A007-1-1, visible light method), glutathione peroxidase (GSH-Px) (A005-1-2, colorimetric method) and quick sperm stain assay kits were purchased from Nanjing Jiancheng Biotech Co., Ltd (Nanjing, China). FastKing gDNA Dispelling RT SuperMix (KR118-02, reverse transcription) and SYBR Green I fluorescent (FP205, chimeric fluorescence method) were obtained from TIANGEN Biotech Co., Ltd (Beijing, China). Bicinchoninic acid (BCA) protein detection kit (PC0020, microplate reader method) was obtained from Solarbio (Beijing, China). Enhanced chemiluminescence (ECL) (P0018S, chemiluminescence method) reagent was from Beyotime (Shanghai, China). Primary antibodies against Keap1 (bs-4900R, 1:500–2000), Nrf2 (bs-1074R, 1:100–1000), NQO1 (bs-2184R, 1:500–2000), HO-1 (bs-2075R, 1:500–2000), Bax (bs-0127R, 1:500–2000), Bcl-2 (bs-0032R, 1:500–2000), Caspase-9 (bs-20773R, 1:500–2000) and FADD (bs-20780R, 1:500–2000) were purchased from Bioss (Beijing, China). Primary antibodies against Fas (AF5342, 1:500–2000) and Fas ligand (FasL) (AF0157, 1:500–3000) were purchased from Affinity Biosciences (Cincinnati, USA). Primary antibodies against Caspase-3 (19677-1-AP, 1:500–2000), Caspase-8 (13423-1-AP, 1:500–1000), beta-actin (20536-1-AP, 1:1000–5000), and secondary antibodies (SA00001-2, 1:2000–10000) were purchased from Proteintech (Wuhan, China).

2.2. Acquisition of the targets of TFA and SeY against testicular damage

The 2D structure, 3D structure and canonical SMILES of TFA and SeY were acquired by PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>, version 2019). The possible targets of TFA and SeY were generated from SwissTargetPrediction (<http://www.swisstargetprediction.ch/>, version 2017), PharmMapper database (<http://www.lilab-ecust.cn/pharmmapper/>), Comparative Toxicogenomics (<http://ctdbase.org/>), BATMAN-TCM database (<http://bionet.ncpsb.org.cn/batman-tcm/>, version 2.0), and Coremine database (<https://www.coremine.com/>). The thresholds for target screening were Norm Fit > 0.7 in PharmMapper, Interaction Count > 1 in Comparative Toxicogenomics, Scored > 15 in BATMAN-TCM, and Significance < 0.001 in Coremine. Next, the possible targets of testicular damage were identified by online databases GeneCards (<https://www.genecards.org/>, version 5.18) through scouring the keyword of “testicular damage”, the threshold for target screening was Relevance > 10. Lastly, the targets of both TFA and SeY against testicular damage were acquired through intersection of the drug and disease targets. All target screening was accessed on 31 October 2023.

2.3. Constructing of protein-protein interaction (PPI) network and enrichment analysis

The PPI of intersection genes was counted by STRING database (<https://string-db.org/>). The file of PPI acquired from STRING was imported into Cytoscape software (version 3.10.0) to establishment the PPI network and used centiscape (version 2.2) to count the topological parameters of PPI network. Then, the Maximum Cross Correlation (MCC) counted by the app plug-in cytoHubba (version 0.1) was used to identify the hub genes of the PPI network. Finally, the PPI network of hub genes was constructed by Cytoscape software. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of hub genes was carried out by the KOBAS 3.0 online database (<http://bioinfo.org/kobas>).

2.4. Animals and experimental design

Forty six-week-old male Kunming mice were procured from the Experimental Animal Center of Yanbian University (Certificate No. SCXK(J) 2011-0007, Yanji, China). This work was conducted in accordance with the Local Guide for the Care and Use of Laboratory Animals of Yanbian University. The mice were raised under standard specific pathogen free (SPF) conditions. All experimental procedure was approved by the Laboratory Animal Ethics Committee of Yanbian University (Approved number: YD20240001, Yanji, China).

Mice were randomly assigned to five groups: control group, ZEA (20 mg/kg) group, SeY (0.4 mg/kg) + ZEA group, TFA (50 mg/kg) + ZEA group, SeY (0.4 mg/kg) + TFA (50 mg/kg) + ZEA group. The dosage of ZEA, TFA and SeY used here was obtained from pre-experimental and previous studies (Lin et al., 2022; Liu et al., 2017). The animals in control group were intragastrically administered with olive oil, and the other groups were given with ZEA at the same time. Then, the animals in control group and ZEA group were intragastrically administered with doubled-distilled water, and the remaining groups were intragastrically administered with SeY, TFA and SeY + TFA once a day for 14 d, respectively. The blood, testis and epididymis tissues of mice were collected and dissected quickly for subsequent experiments.

2.5. Sperm concentration, motility and malformation

One epididymis of each mice was cut into small pieces in preheated physiological saline and incubated at 37°C for 15 min to obtain sperm suspension. The sperm concentration was counted. The sperm motility was detected by computer-assisted semen analysis (CASA) system. At the same time, the sperm suspension was smeared on a slide and stained with a rapid sperm staining kit, then the sperm morphology was examined under a microscope and the abnormal sperm in head, neck/mid-piece, and tail were recorded and the malformation rate of sperm was calculated.

2.6. Detection of reproductive hormones and inflammatory cytokines in sera

The hormone LH, FSH and testosterone, and inflammatory parameter TNF- α , IL-6, and IL-1 β levels in mouse sera were determined by ELISA kits. Standard operation was carried out in strict accordance with the manufacture's instructions and the absorbance was measured by a micro plate reader, standard curves were made and the levels of hormones and inflammatory cytokines were calculated.

2.7. Measurement of oxidative stress biomarkers in testis

The testis tissues were collected and homogenized, and the total protein concentration was measured by BCA. The levels of MDA, SOD, CAT and GSH-Px in testis were measured according to the kit's instructions. Meantime, ROS level was analyzed following ROS kit's instructions: testis tissues were prepared into cell suspension, the cell suspension was centrifuged, the precipitate was washed and resuspended with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), and incubated at 37°C for 30 min, the cells were photographed under a fluorescence microscope, the fluorescence value was measured by a fluorescence spectrophotometer and ROS content was calculated.

2.8. Histopathology and ultrastructure observation

The testis tissues were fixed in 4 % paraformaldehyde for 48 h and embedded in paraffin. The specimens were sectioned into 5 μ m thickness and stained with hematoxylin and eosin (H&E), and the histopathological changes of mouse testis were examined under a light microscope. In addition, the testis tissues were cut into 1 mm³ size and fixed in the transmission electron microscope (TEM) fixative. After washing with

PBS, the tissues were fixed in 1 % osmic acid solution for 2 h. Then the fixed tissues were dehydrated with graded concentrations of ethanol and acetone, coated with an embedding agent. The sections were cut into thin slices and stained with uranyl acetate-lead citrate for 15 min. The ultrastructure of testis tissues was observed by a TEM.

2.9. TUNEL assay

The paraffin samples of testis were dewaxed, hydrated, and washed with PBS, and added with proteinase K working solution and incubated at 37°C for 30 min. The samples were confined with sealing solution for 10 min at room temperature, incubated with TUNEL working solution for 1 h at 37°C and added DAPI staining solution in the dark at room temperature for 5 min. Finally, the samples were washed and observed by a fluorescence microscope.

2.10. Immunohistochemistry

The paraffin samples of testis were sliced into 5 μ m thickness. Each section was dewaxed, rehydrated, and antigenically repaired with citric acid antigen repair buffer. After blocking endogenous peroxidase with 3 % hydrogen peroxide solution, the testicular section was sealed with 3 % bovine serum albumin (BSA), cultivated overnight at 4°C with specific primary antibody anti-Keap1, Nrf2, Caspase-3, and Caspase-8. The section was incubated with secondary antibodies, and developed with a newly prepared 3,3'-Diaminobenzidine (DAB) staining solution. Finally, the section was re-stained with hematoxylin and then dehydrated. The images were photographed and analyzed with Image J software.

2.11. Western blot

The proteins from testis tissues were extracted using RIPA lysate buffer and the protein concentration was measured by BCA. Equal amounts of protein were mixed with 2 $\times$ loading buffer, separated by 12–15 % SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5 % skim milk for 1 h at room temperature and cultivated with specific primary antibodies anti-Keap1, Nrf2, HO-1, NQO1, Bax, Bcl-2, Caspase-3, Caspase-9, Fas, FasL, FADD, Caspase-8, and β -actin at 4°C for overnight. The membranes were washed with TRIS-buffered saline with 2 % Tween (TTBS), and incubated with secondary antibodies at room temperature for 1 h. The blots were detected using ECL detection system and blot densities were analyzed using Image J software.

2.12. Statistical analysis

The experimental data were expressed as means $\pm$ standard deviation (Means $\pm$ SD). Prim 9.0 software (GraphPad, USA) was used for the statistical analysis. One-way ANOVA was used to analyze, and the measurements were considered significantly different when $P < 0.05$ or $P < 0.01$.

3. Results

3.1. Targets of TFA combined with SeY against testicular damage

Potential targets of TFA combined with SeY and testicular damage were identified by an online database. About 417 target genes of TFA combined with SeY and 316 target genes of testicular damage were identified. Subsequently, the targets of TFA combined with SeY were intersected with the targets of testicular damage and 36 intersection genes were obtained, including *IL1B*, *CAT*, *GSTP1*, *TP53*, *AR*, *SOD1*, *DDIT3*, *FUS*, *MAPK1*, *TGFB1*, *VIM*, *CCND1*, *BCL2*, *CASP3*, *PARP1*, *CASP9*, *CDKN1A*, *CDKN1B*, *ESR1*, *TNF*, *TOP1*, *BAX*, *ESR2*, *CDK2*, *CYP19A1*, *CHEK1*, *CCNA2*, *ALB*, *STS*, *SHBG*, *HSP90AA1*, *GSR*, *MAPK8*,

MGMT, *ABL1*, *KIT* (Supplementary Material Fig. S1). These genes were the potential targets of TFA combined with SeY against testicular damage.

3.2. Construction of PPI network for potential targets of TFA combined with SeY against testicular damage

The intersection targets of TFA combined with SeY and testicular damage were used to construct the PPI network composed of 36 nodes and 343 edges, with an average node degree of 19.1 (Fig. 1A). The color from dark to light showed that the node degree value was from high to low, and the node size from large to small showed that the node degree value was from high to low. Based on MCC values, the top 10 targets of TFA combined with SeY against testicular damage were *TP53*, *CASP3*, *BCL2*, *CCND1*, *TNF*, *ESR1*, *HSP90AA1*, *PARP1*, *CDKN1A*, and *TGFB1*. The PPI network of hub targets had 10 nodes and 45 edges, with an average node degree of 28 (Fig. 1B).

3.3. GO and KEGG pathway enrichment analysis

KOBAS 3.0 database was used to further conduct GO and KEGG enrichment analysis of top 10 collective target genes. The enrichment analysis results were screened, and the filtering condition was P -value < 0.001 .

The top 30 terms of GO enrichment were selected and displayed in Fig. 1C. The results showed that the targets of TFA combined with SeY against testicular damage involved 20 biological processes (BP), including cytokine-mediated signaling pathway, cellular response to DNA damage stimulus, negative regulation of transcription by RNA polymerase II, response to X-ray, response to estradiol, response to drug, positive regulation of protein phosphorylation, positive regulation of protein kinase B signaling, positive regulation of peptidyl-serine phosphorylation, negative regulation of production of miRNAs involved in gene silencing by miRNA, negative regulation of gene expression, negative regulation of cell growth, response to glucocorticoid, response to gamma radiation, response to antibiotic, positive regulation of pri-miRNA transcription by RNA polymerase II, positive regulation of neuron apoptotic process, neuron apoptotic process, mitochondrial DNA repair, B cell lineage commitment, nucleus, nucleoplasm, protein-containing complex, identical protein binding, protein kinase binding, enzyme binding, ubiquitin protein ligase binding, transcription factor binding, protease binding, histone deacetylase binding.

The top 30 terms of KEGG pathway enrichment were chosen and exhibited in Fig. 1D. The results showed that the targets of TFA

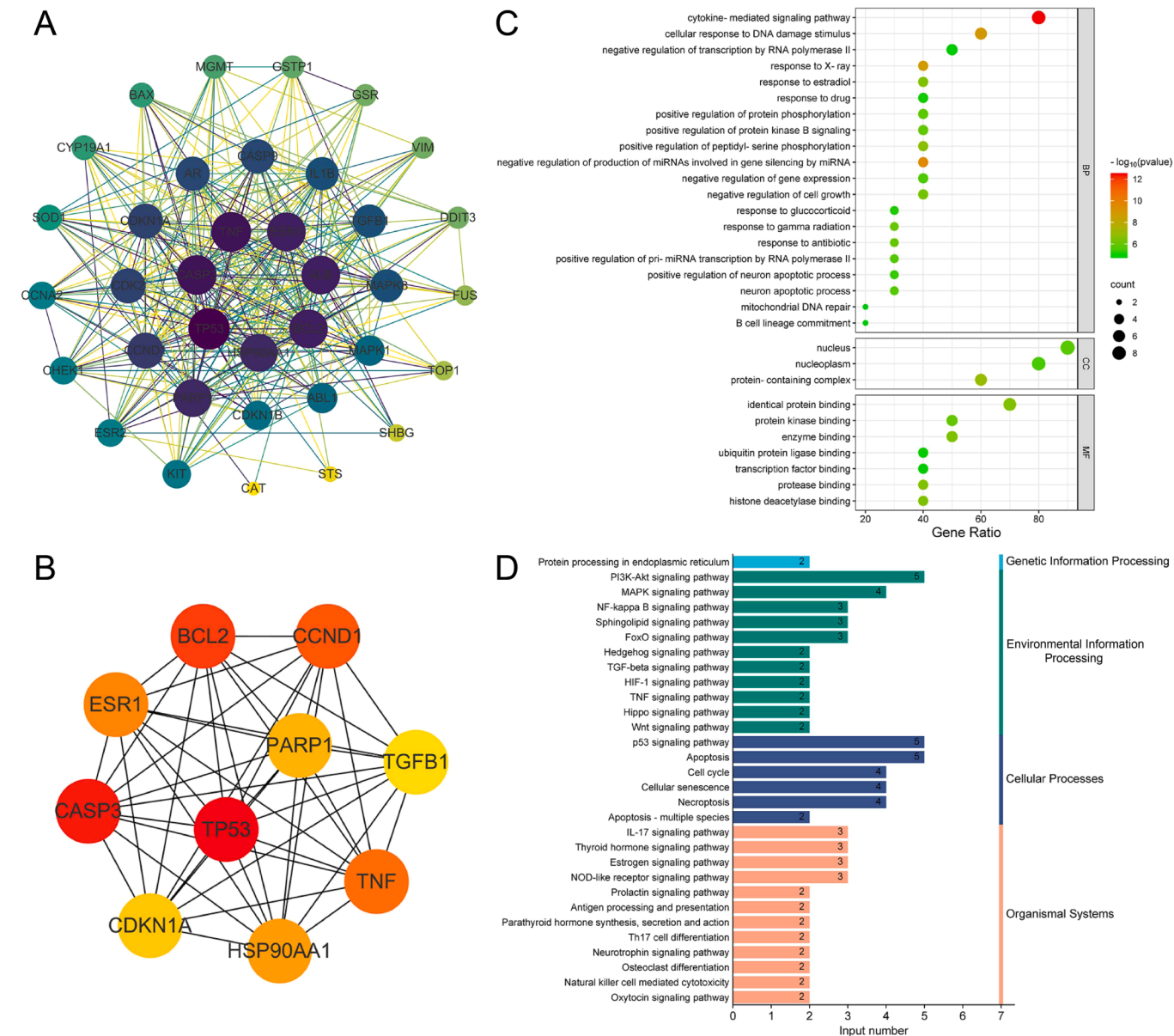


Fig. 1. PPI analysis and screening of the targets, GO enrichment and KEGG pathway analysis of key target genes. (A) Establishment of PPI network by collective targets. (B) MCC algorithm confirmed the hub genes and constructed the PPI network. The node size from large to small and the color from dark to light mean that the node degree value from high to low. (C) The top 30 terms of GO enrichment analysis. The size of the dot indicates the number of enriched genes, and the color of the dot indicates the P -value. (D) The 30 terms of KEGG pathway enrichment analysis.

combined with SeY against testicular injury were mainly enriched in environmental information processing, including PI3K/AKT1, MAPK, NF- κ B and other signaling pathways. And these target genes were also involved in the cellular processes of p53 signaling pathway, apoptosis, cell cycle, etc. Moreover, these target genes were mainly involved in organismal systems, including IL-17, thyroid hormone, estrogen and other signaling pathways, and 1 genetic information processing.

3.4. Effect of TFA combined with SeY against ZEA-induced testicular damage in mice

To evaluate the protective effect of TFA combined with SeY on ZEA-induced testicular damage, the pathological and ultrastructural changes of testis were observed. The histopathological evaluation was shown in Fig. 2A, the testis of control group was structurally intact, and the seminiferous tubules were arranged neatly and tightly. In ZEA group,

the testicular seminiferous tubules were irregularly shaped and loosely arranged, spermatozoa and interstitial cells were reduced and damaged, the tubular lumen was obstructed, and spermatogonial cells in the lumen were detached; however, the drug treatment alleviated these pathological changes. Among them, the SeY+TFA group showed the best effect and the restoration of testicular structure was essentially same as that in control group.

The TEM results of testis were exhibited in Fig. 2B, in control group, the testicular supporting cells were morphologically normal with pike-shaped mitochondria, clear bilayer membrane structure and mitochondrial cristae folds, and a structurally intact BTB with tight junctions between the supporting cells. However, in ZEA group, testicular supporting cells were severely damaged, mitochondria were swollen and deformed, bilayer membrane structure and mitochondrial cristae were unclear or even vacuolated, and BTB structure was blurred and incoherent or even fractured. Compared with ZEA group, TFA and SeY alone

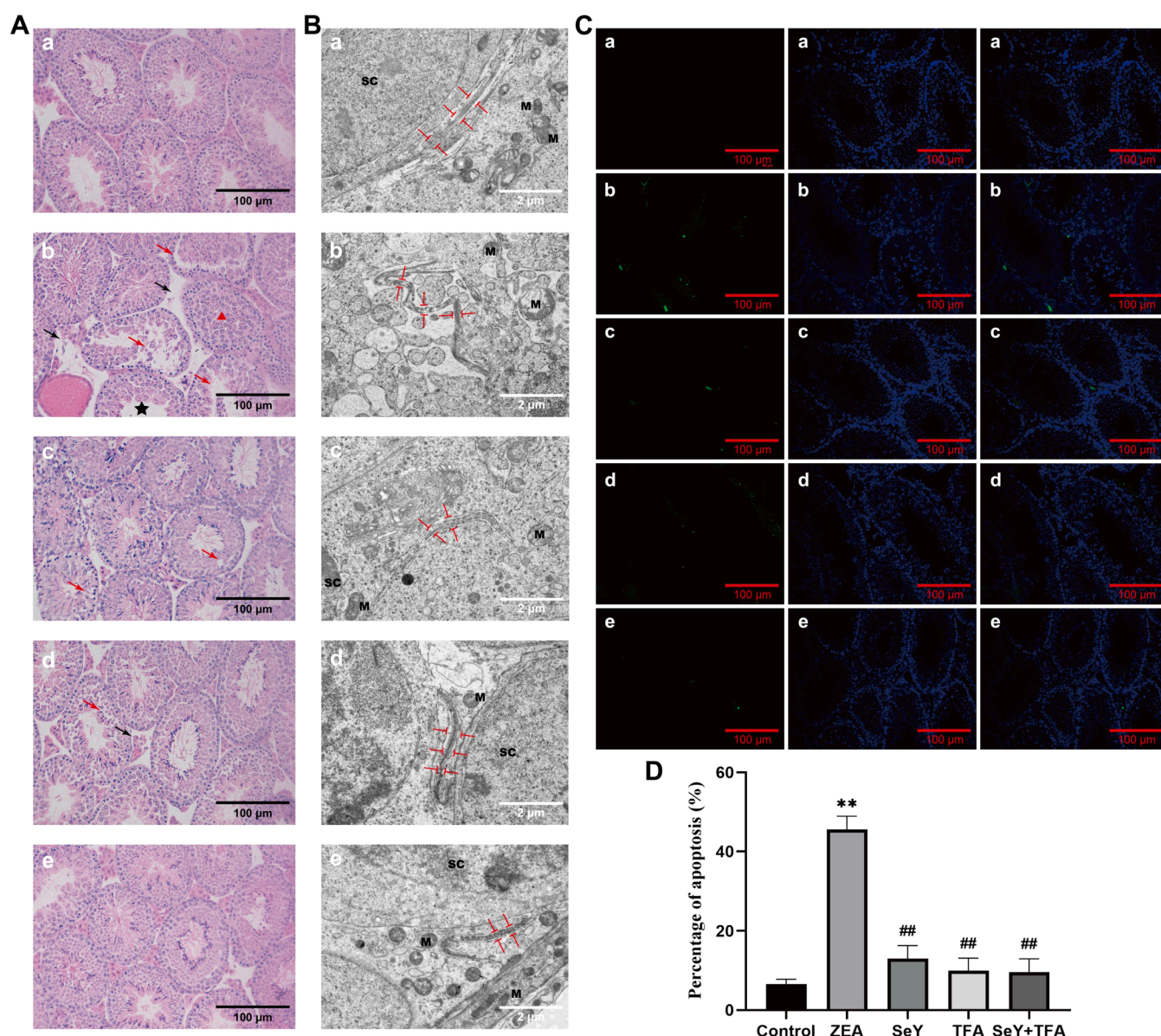


Fig. 2. TFA combined with SeY alleviated ZEA-induced testicular injury of mice. (A) Histological observation of H&E staining (200 $\times$, red arrows: luminal cell detachment, black arrows: intercellular vacuolisation, red triangles: luminal obstruction, black pentagrams: reduced mature spermatozoa). (B) Ultrastructure observation of testis (8,000 $\times$, SC: support cells, M: mitochondria, red arrow: blood-testis barrier). (C) The testicular apoptosis was determined by TUNEL. (D) The rates of testicular apoptosis. Results were expressed as Means $\pm$ SD (n = 6). ** P < 0.01 vs. control group; ## P < 0.01 vs. ZEA group. (a) control group, (b) ZEA group, (c) SeY group, (d) TFA group, (e) SeY+TFA group.

or in combination could alleviate the above pathological changes to different degrees, and the combined treatment group had the best recovery effect.

To further investigate the extent of testicular damage, we examined testicular apoptosis. As exhibited in Fig. 2C-D, compared to control group, the rate of apoptosis in ZEA group was highly significantly up-regulated ($P < 0.01$), up to 46 %. Compared to ZEA group, the rates of apoptosis in SeY, TFA and SeY+TFA groups were significantly lowered to 13 %, 10 % and 9 %, respectively ($P < 0.01$).

3.5. Effect of TFA combined with SeY on ZEA-induced sperm quality and hormone levels in mice

The sperm parameters and sex hormone levels of mice were further examined, as exhibited in Fig. 3A-C, exposed to ZEA, a significant decrease in sperm count and motility and a significant increase in sperm malformation rate were observed as compared with control group ($P < 0.01$). While the sperm count and motility in SeY, TFA and SeY+TFA groups were significant increased ($P < 0.01$) and the sperm malformation rate in these groups were lower than those in ZEA group ($P < 0.01$). By comparison, the improvement of sperm quality in combined treatment group was more obvious than that in alone treatment group ($P < 0.01$).

The hormone levels in serum were shown in Fig. 3D-F. Compared to control group, the levels of testosterone and LH in ZEA group were remarkably reduced ($P < 0.01$), and ZEA could also decreased the level of FSH, but was not significant compared to control group ($P > 0.05$). Compared to ZEA group, the levels of testosterone and LH in SeY, TFA and SeY+TFA groups were remarkably enhanced ($P < 0.05$ or $P < 0.01$), the levels of FSH in all treatment groups were enhanced but no statistical significance ($P > 0.05$). By comparison, the effect of SeY+TFA group was better than that of SeY or TFA alone group ($P < 0.05$ or $P < 0.01$).

3.6. Effect of TFA combined with SeY on ZEA-induced testicular oxidative stress and inflammation in mice

The antioxidant capacity was investigated by determining the levels of oxidative biomarker ROS, MDA, SOD, CAT, and GSH-Px. As shown in Fig. 4A-E, compared to control group, the levels of ROS and MDA in ZEA group were highly up-regulated ($P < 0.01$), and the levels of SOD, CAT, and GSH-Px were significantly down-regulated ($P < 0.01$). However, after treatment, the levels of ROS and MDA were significantly down-regulated and the levels of SOD, CAT, and GSH-Px were significantly up-regulated in SeY, TFA and SeY+TFA groups ($P < 0.05$ or $P < 0.01$), and it was found that drug combination treatment was more effective than drug alone treatment ($P < 0.05$).

Further, the secretion of inflammatory indicator TNF- α , IL-6, and IL-1 β in mouse sera was examined. As shown in Fig. 4F-H, compared with control group, the levels of TNF- α , IL-6, and IL-1 β in ZEA group were highly significantly increased ($P < 0.01$). After treatment, the levels of TNF- α , IL-6, and IL-1 β in SeY, TFA and SeY+TFA groups were significantly decreased ($P < 0.01$). Drug combination treatment group had a better anti-inflammatory effect than drug alone treatment group ($P < 0.05$ or $P < 0.01$).

3.7. Effect of TFA combined with SeY on Keap1/Nrf2/HO-1 signaling pathway

The Keap1/Nrf2/HO-1 pathway plays a crucial role in the development of oxidative stress. To explore the anti-oxidative stress of TFA combined with SeY by the Keap1/Nrf2/HO-1 signaling pathway in ZEA-induced testicular damage, the expression of the pathway key proteins was detected by Western blot. As exhibited in Fig. 5A-E, compared to control group, the levels of Nrf2, NQO1, and HO-1 in ZEA group were remarkably decreased ($P < 0.01$), and the levels of Keap1 were remarkably elevated ($P < 0.01$). Compared to ZEA group, TFA and SeY alone or in combination obviously enhanced the expression of Nrf2, NQO1, and HO-1 and reduced the level of Keap1 ($P < 0.05$ or $P < 0.01$), and it was also found that the combined treatment had a better

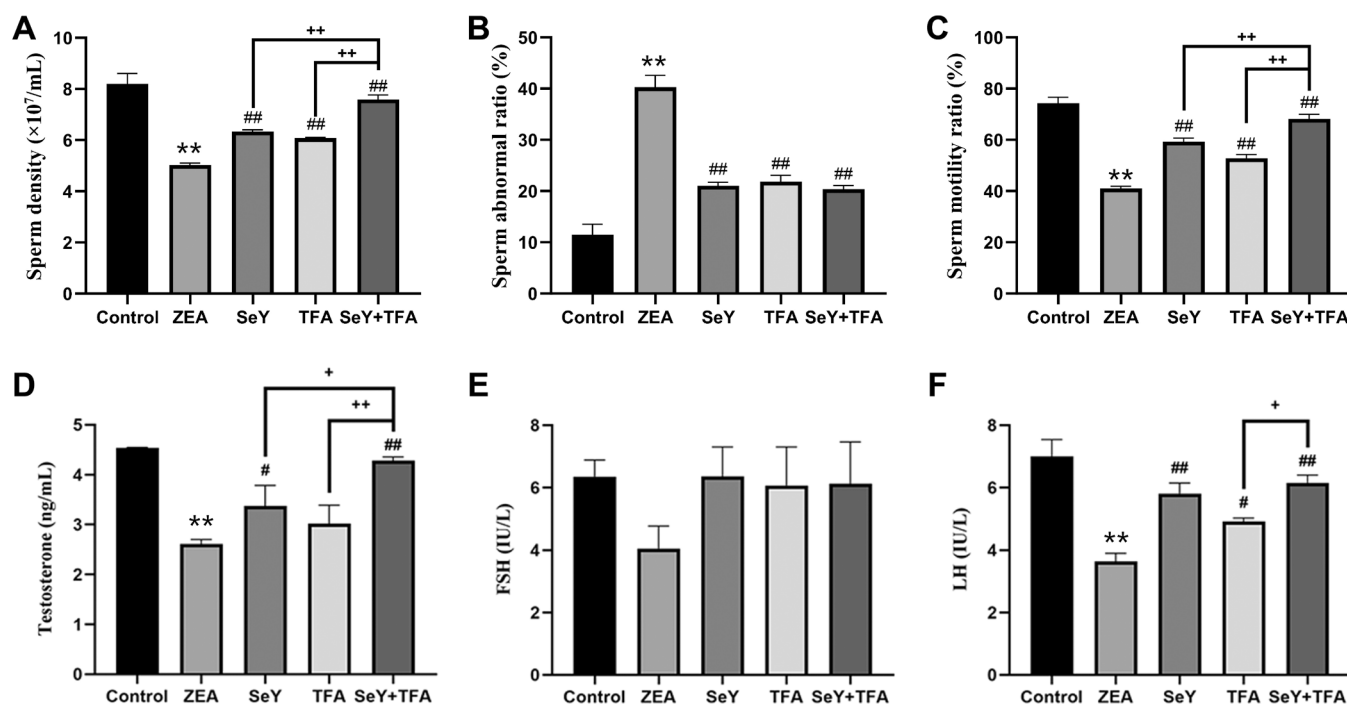


Fig. 3. TFA combined with SeY improved sperm quality and hormone levels in ZEA-induced reproductive injury of male mice. (A) Sperm density. (B) Sperm abnormal ratio. (C) Sperm motility. (D) Testosterone content. (E) FSH content. (F) LH content. Results were expressed as Means $\pm$ SD ($n = 6$). ** $P < 0.01$ vs. control group; # $P < 0.05$, ## $P < 0.01$ vs. ZEA group; + $P < 0.05$, ++ $P < 0.01$ vs. SeY and TFA groups.

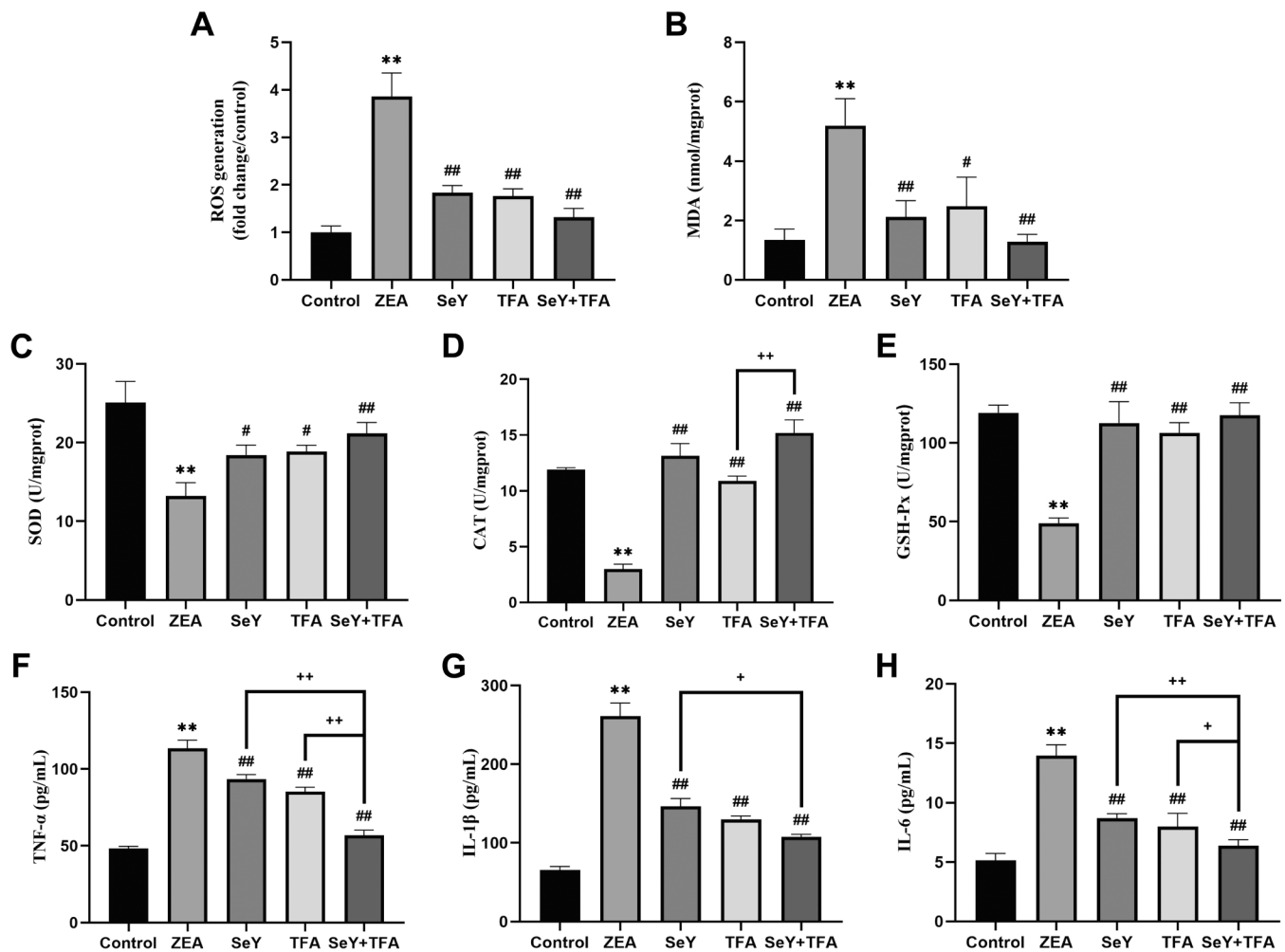


Fig. 4. TFA combined with SeY alleviated ZEA-induced testicular oxidative stress of mice. (A-E) The levels of oxidative biomarker ROS, MDA, SOD, CAT, and GSH-Px. (F-H) The secretion of inflammatory cytokine TNF α , IL-1 β , IL-6. Results were expressed as Means $\pm$ SD (n = 6). (a) control group, (b) ZEA group, (c) SeY group, (d) TFA group, (e) SeY+TFA group. ** P < 0.01 vs. control group; # P < 0.05, ## P < 0.01 vs. ZEA group; + P < 0.05, ++ P < 0.01 vs. SeY and TFA groups.

regulatory role on the Keap1/Nrf2/HO-1 pathway than the alone treatment (P < 0.05 or P < 0.01).

3.8. Effect of TFA combined with SeY on mitochondrial and death receptor signaling pathways

The mitochondrial and death receptor pathways are the two main pathways of apoptosis, which are regulated through endogenous and exogenous pathways, respectively. The expression of the apoptosis-related protein Bcl-2, Bax, cleaved Caspase-3, cleaved Caspase-9, Fas, FasL, FADD, and cleaved Caspase-8 was detected by Western blot. As shown in Fig. 6A-B, compared to control group, the expression of Bax, cleaved Caspase-3, cleaved Caspase-9, Fas, FasL, FADD, and cleaved Caspase-8 in ZEA group was remarkably up-regulated (P < 0.01), and the expression of Bcl-2 was significantly down-regulated (P < 0.01). Compared with ZEA group, TFA and SeY alone or in combination obviously down-regulated the expression of Bax, cleaved Caspase-3, cleaved Caspase-9, Fas, FasL, FADD, and cleaved Caspase-8 and up-regulated the expression of Bcl-2 (P < 0.05 or P < 0.01), and it was also found that the combined treatment had a better regulatory role on the mitochondrial and death receptor pathways than the alone treatment.

4. Discussion

Excessive or prolonged consumption of ZEA-containing feed and food may cause damage to many organs and physiological systems of the body, among which ZEA causes the most serious damage to the reproductive system (Cai et al., 2024). TFA, a class of bioactive substances extracted from the traditional Chinese medicine *Astragalus membranaceus* Bunge, possesses a variety of biological and pharmacological activities and plays a protective role against various diseases (Caglayan et al., 2022). Se is an essential trace element for human and animal health, widely involved in various physiological and biochemical activities with antioxidant, anti-inflammatory and immunomodulatory effects (Genchi et al., 2023). Studies have shown that drug combination has the advantages of improving efficacy, mitigating side effects, and delaying disease progression in the treatment of diseases (Wang et al., 2022a; Ling et al., 2018). However, it is not clear whether the combination of traditional Chinese medicine and trace elements has a better therapeutic effect on reproductive injury caused by ZEA, this study was the first to investigate the effect of the combination of TFA and SeY on reproductive injury and its mechanism in male mice exposed to ZEA. It demonstrated that TFA combined with SeY synergistically protected against ZEA-induced reproductive injury through anti-inflammatory, antioxidant, and anti-apoptotic mechanisms in male mice.

Network pharmacology is an integrative approach that combines traditional pharmacology with modern bioinformatics, big data

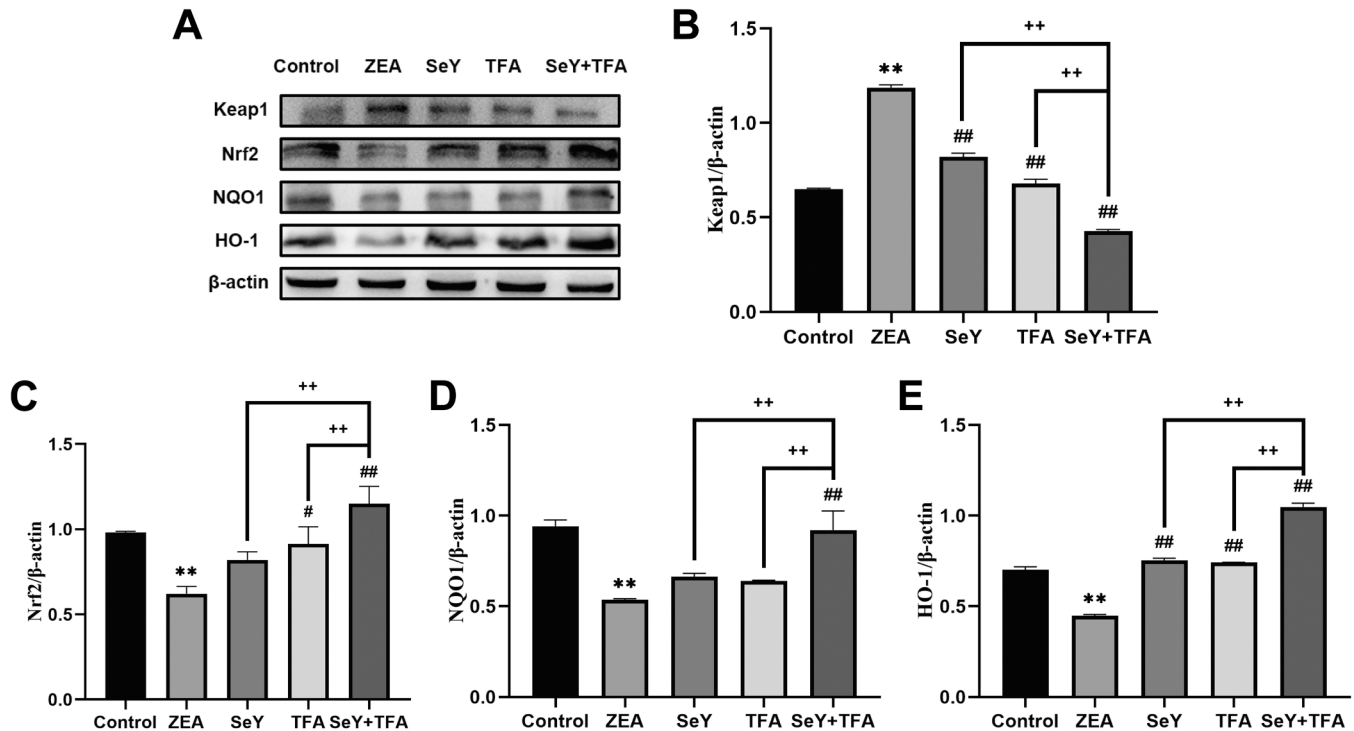


Fig. 5. TFA combined with SeY activated Keap1/Nrf2/HO-1 signaling pathway in ZEA-induced male mice. The protein expression of Keap1, Nrf2, NQO1, and HO-1 was detected by Western blot and β -actin as internal control. Results were expressed as Means $\pm$ SD ($n = 4$). ** $P < 0.01$ vs. control group; # $P < 0.05$, ## $P < 0.01$ vs. ZEA group; ++ $P < 0.01$ vs. SeY and TFA groups.

analytics, and network biology to systematically explore the mechanisms and effects of drugs within biological systems (Nogales et al., 2022). This paradigm enhances the efficiency and success rate of drug discovery and plays an important role in addressing the treatment of complex diseases. Therefore, in this study, we first utilized network pharmacology to screen 417 targets of TFA combined with SeY and 316 targets of testicular damage, and obtain 36 candidate targets by their intersections, and screen 10 hub targets for subsequent enrichment analysis (Fig. S1). Based on GO and KEGG enrichment analysis, we speculated that the protective effect of TFA combined with SeY against ZEA-induced reproductive injury in male mice might be related to its modulatory effects on oxidative stress, inflammatory response and apoptosis (Fig. 1). Subsequently, we carried out *in vivo* experiment in male mice to verify this speculation.

The testis is one of the most important reproductive organs in male mammals, which is involved in spermatogenesis and androgen secretion. It has been found that oral administration of ZEA resulted in damage to testicular structure in male mice, as evidenced by disorganized seminiferous tubule arrangement and atrophy (Lin et al., 2022), which was consistent with our H&E staining results (Fig. 2A). In addition, TUNEL assay showed that ZEA caused testicular apoptosis. However, TFA and SeY alone or in combination significantly ameliorated these pathologic changes induced by ZEA, as well as significantly attenuated ZEA-induced apoptosis of testicular tissues (Fig. 2C-D). Spermatogenesis is regulated by a variety of hormones, such as testosterone, FSH, and LH, which interact to ensure normal physiological function of the testis (Mararajah et al., 2024). Testosterone is an androgen produced by testicular mesenchymal stromal cells, exerting a crucial role in the development and maintenance of the male reproductive system (Salonia et al., 2019). Additionally, testosterone is essential for sustaining the environment necessary of sperm production and maintaining the integrity of BTB (Smith et al., 2014). FSH and LH are hormones secreted by the anterior pituitary gland. FSH stimulates spermatogenesis and maturation, while LH acts on testicular interstitial cells to promote the production and secretion of testosterone

(Ramaswamy et al., 2015). BTB is a physical barrier within the mammalian testis that prevents the diffusion of various endogenous and exogenous toxic substances (Stanton et al., 2016). Previous research has documented that ZEA could cause reproductive disorders in males, severely impede spermatogenesis, destroy the structure of BTB, and ultimately lead to sterility (Lin et al., 2022). Meanwhile, it has been showed that ZEA exposure decreased the levels of serum FSH and LH in mice (Tan et al., 2020), which was consistent with our present findings about sperm quality and hormone levels. Our study showed that TFA combined SeY could mitigate the decline in sperm quality, enhance serum levels of testosterone, FSH, and LH (Fig. 3), and repair the damaged BTB structure induced by ZEA (Fig. 2B). These findings provided robust evidence that TFA combined with SeY attenuated ZEA-induced reproductive toxicity by improving sperm quality, promoting serum hormone secretion, and restoring the reconstruction of BTB integrity.

Inflammation represents a fundamental protective response of the immune system, aimed at preserving tissue integrity and preventing further damage. Various inflammatory pathologies are associated with the dysregulated overproduction of pro-inflammatory mediators (Frangogiannis et al., 2022; Zhang et al., 2024). Key inflammatory mediators, such as ILs, TNF and IFN play crucial roles in the pathogenesis of various conditions. These mediators exert their biological effects through binding to specific cell surface receptors, thereby orchestrating and amplifying inflammatory response. ZEA has been demonstrated to induce inflammatory damage in various tissues (Ammar et al., 2023; Fan et al., 2018). Upon systemic absorption and metabolism in the gastrointestinal tract, ZEA triggers intestinal inflammation and an elevation of pro-inflammatory mediators in bloodstream (Wang et al., 2024). Subsequently, ZEA, its metabolites, and a range of inflammatory cytokines are transported to the reproductive organs via the circulatory system, where they contribute to reproductive toxicity (Wang et al., 2022). Our findings corroborate this sequence of events. Conversely, TFA combined with SeY effectively reduced serum inflammatory factor levels (Fig. 4F-H). Network pharmacology analysis

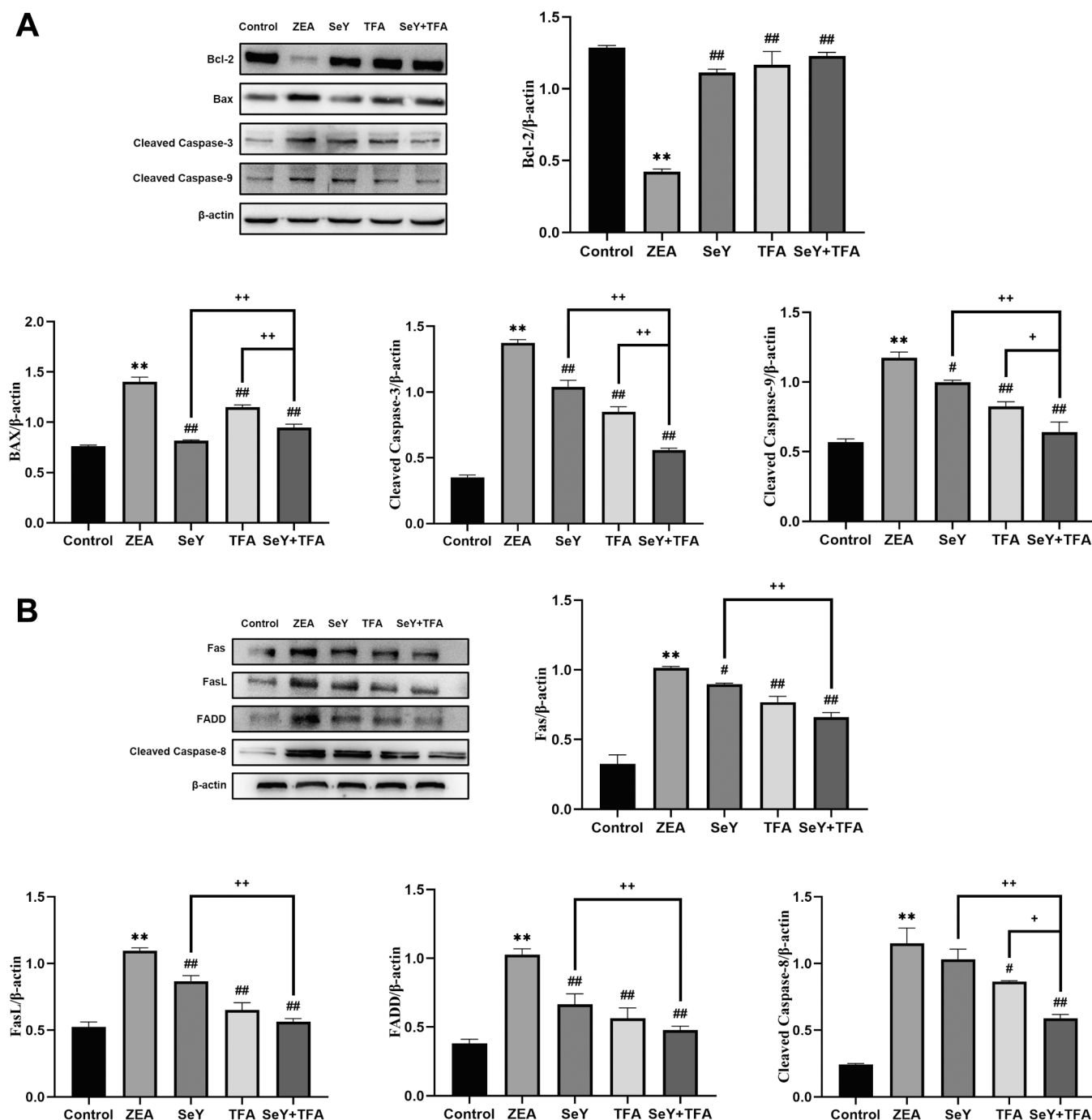


Fig. 6. TFA combined with SeY inhibited mitochondrial and death receptor apoptotic signaling pathways in ZEA-induced male mice. (A) The protein expression of Bcl-2, Bax, cleaved Caspase-3, and cleaved Caspase-9 in mitochondrial apoptotic pathway was detected by Western blot and β -actin as internal control. (B) The protein expression of Fas, FasL, FADD, and cleaved Caspase-8 in death receptor pathway was measured by Western blot and β -actin as internal control. Results were expressed as Means $\pm$ SD ($n = 4$). ** $P < 0.01$ vs. control group; # $P < 0.05$, ## $P < 0.01$ vs. ZEA group; + $P < 0.05$, ++ $P < 0.01$ vs. SeY and TFA groups.

suggested that TFA combined with SeY may exert anti-inflammatory effects by modulating MAPK and NF- κ B signaling pathways. However, the precise mechanisms underlying these effects needs to be studied further.

It was reported that exposure to ZEA significantly induced oxidative stress in goat support cells by promoting an increase of ROS level (Liu et al., 2023). The accumulation of ROS initiates lipid peroxidation, which disrupts the antioxidant defense system and leads to an elevated MDA concentration (Zorov et al., 2014). Maintenance of intracellular redox balance is mediated by several key enzymatic antioxidants, including CAT, SOD, and GSH-Px, which together form a critical

intracellular defense mechanism against oxidative damage (Pisoschi et al., 2015). It has been shown that ZEA exposure increases MDA level and reduces SOD and GSH-Px activity in mouse testicular tissue (Long et al., 2017). Consistent with these findings, our findings showed the same trend, while TFA combined with SeY significantly decreased oxidation levels and enhanced antioxidant activity (Fig. 4A-E), it is also consistent with our network pharmacology analysis. The Keap1/Nrf2/HO-1 signaling pathway is a crucial anti-oxidative stress pathway in mammalian cells, playing a pivotal role in regulating cellular antioxidant defense and providing cell protection (Ma et al., 2013). The Keap1/Nrf2/HO-1 pathway is also a primary target for various drugs

and natural antioxidants to enhance cellular antioxidant capacity. It is demonstrated that ZEA induces oxidative stress in porcine testicular Sertoli cells by inhibiting Nrf2/HO-1 expression (Cao et al., 2021). In our study, ZEA exposure inhibited Nrf2/HO-1 pathway through upregulating Keap1 expression and downregulating Nrf2, HO-1, and NQO1 expression, resulting in oxidative stress in mouse testicular tissues. However, TFA combined with SeY activated Nrf2/HO-1 pathway by regulating these key protein expressions (Fig. 5).

In mammalian cells, apoptosis is categorized into intrinsic apoptosis and extrinsic apoptosis based on the distinct mechanisms (Yuan et al., 2024). Extrinsic apoptosis is initiated by the activation of death receptors on the plasma membrane, while intrinsic apoptosis is triggered by mitochondrial stress response. The extrinsic apoptotic pathway is initiated when the death receptor Fas binds to its ligand FasL, leading to the activation of key effector protein Caspase-8 in this pathway. Activated Caspase-8 triggers a downstream cascade of caspases, culminating in apoptosis (Holloway et al., 2024). In addition, Caspase-8 can cleave and activate Bcl-2 family proteins, promoting interactions within this family that increase the permeability of the outer mitochondrial membrane. This permeability change activates the intrinsic apoptotic pathway by releasing cytochrome c from the mitochondria, which subsequently activates Caspase-9, further amplifies the apoptotic response. Studies have found that ZEA can cause apoptosis in reproductive system through the death receptor and mitochondrial apoptotic pathway (Xu et al., 2016; Wu et al., 2021). Our findings also exhibited that ZEA activated the expression of key proteins associated with both the mitochondrial and death receptor pathways to induce apoptosis in testis. Notably, our Network pharmacology results suggested that the targets of TFA combined with SeY, such as *Bax*, *Bcl-2*, *CASP3*, and *CASP9*, were linked to apoptotic modulation, the *in vivo* experiment further demonstrated that TFA combined with SeY regulated these key protein expression levels in testicular tissue (Fig. 6). It indicated that TFA combined with SeY attenuated ZEA-induced apoptosis in mouse testis through inhibiting the mitochondrial and death receptor apoptotic pathways.

5. Conclusion

In this study, we explored the effects and mechanisms of TFA combined with SeY on ZEA-induced testicular injury in mice by network pharmacology and *in vivo* experiment. The results demonstrated that TFA and SeY alone or in combination provided protective effects against ZEA-induced testicular injury, in which the combined treatment had the most pronounced efficacy. Specifically, TFA combined with SeY mitigated ZEA-induced excessive inflammation, oxidative stress and apoptosis in testicular tissue by modulating Keap1/Nrf2/HO-1, mitochondrial apoptotic and death receptor signaling pathways. Our findings provides valuable insights for the combined use of traditional Chinese medicine and trace elements in controlling reproductive toxicity of ZEA.

CRediT authorship contribution statement

Tian Yu: Visualization, Formal analysis. **Xue-mei Zhang:** Writing – review & editing, Supervision, Resources, Project administration. **Rui Sang:** Methodology, Formal analysis, Data curation. **Xin-man Liu:** Methodology, Formal analysis. **Wei Wang:** Writing – original draft, Validation, Methodology, Data curation. **Bing-jie Ge:** Visualization, Methodology, Conceptualization.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2025.118798.

Data availability

Data will be made available on request.

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