

Original Article

Asian Pacific Journal of Reproduction

Journal homepage: <https://journals.lww.com/apjr/>

doi: 10.4103/apjr.apjr_222_25

Protective effect and mechanism of alpha-lipoic acid on lead-induced Sertoli cell damage in rooster testes

Junrong Li^{1✉}, Shanli Zhu^{1,2}, Nanwei Ye², Fangxiong Shi²¹College of Agriculture, Jinhua University of Vocational Technology, Jinhua, 321000, China²College of Animal Science and Technology, Nanjing Agricultural University, Nanjing 210095, China

ABSTRACT

Objective: To investigate the protective effect of alpha-lipoic acid on lead-induced Sertoli cell injury and its potential molecular mechanisms using primary Sertoli cells from rooster testes.

Methods: Primary Sertoli cells from 6-week-old rooster testes were divided into four groups. They were treated with serum-free DMEM-F12 medium (the control group), 40 μ M lead acetate, 100 μ M alpha-lipoic acid, or 40 μ M lead acetate plus 100 μ M alpha-lipoic acid for 24 h, respectively. Cell viability, reactive oxygen species levels (DCFH-DA), antioxidant enzyme activities (catalase, superoxide dismutase), blood-testis barrier-related proteins, nuclear factor-erythroid 2-related factor 2 (Nrf2) pathway components, proliferation/apoptosis markers (Western blot), and related gene expression (qPCR) were analyzed.

Results: Alpha-lipoic acid treatment significantly reduced lead-induced reactive oxygen species (ROS) production by 58.3% compared to the lead acetate group ($P < 0.05$), enhanced catalase activity by 35.8% ($P < 0.05$), and elevated lead-reduced PCNA expression by 47.2% ($P < 0.05$). Lead acetate 40 μ M plus 100 μ M alpha-lipoic acid significantly increased Nrf2 expression by 78.9% and *HO1/NQO1* expression by 97.5% and 89.4%, respectively ($P < 0.05$), significantly upregulated the expression of Bcl2 protein and significantly downregulated Bax protein expression ($P < 0.05$).

Conclusions: Alpha-lipoic acid enhances the antioxidant properties of Sertoli cells by activating the Nrf2 pathway, restores lead-induced excessive autophagy, and inhibits apoptosis, thus alleviating the damage caused by lead in Sertoli cells.

KEYWORDS: Alpha-lipoic acid; Lead; Sertoli cells; Blood-testis barrier; Nrf2

1. Introduction

Lead (Pb) is widely used and can induce serious damage in reproduction, and may cause lead-induced testicular toxicity[1]. Research has shown that lead induces excessive reactive oxygen

species (ROS) production, disrupts the antioxidant system, and causes oxidative stress[2]. Huang *et al*[3] demonstrated that lead induces oxidative stress in both germ cells and stromal cells, further triggering defense responses, including heat shock and autophagy, which lead to cellular damage and reproductive dysfunction in chickens. In our previous study, we found that lead induces

Key Points

Question: Can alpha-lipoic acid protect against lead-induced damage to Sertoli cells and what are the underlying molecular mechanisms?

Results: In this *in vitro* study using primary rooster Sertoli cells, alpha-lipoic acid treatment significantly reduced lead-induced reactive oxygen species production by 58.3%, enhanced catalase enzyme activity by 35.8%, and elevated Nrf2 protein expression by 78.9% compared to lead-treated cells. Alpha-lipoic acid also preserved blood-testis barrier-related protein expression and reduced autophagy markers.

Meaning: Alpha-lipoic acid protects Sertoli cells from lead toxicity by activating the Nrf2 antioxidant pathway, restoring blood-testis barrier integrity, and preventing excessive autophagy, suggesting potential therapeutic applications for lead-induced reproductive toxicity.

✉ To whom correspondence may be addressed. E-mail: lijornsky@163.com

This is an open access article distributed under the terms of the Creative Commons Attribution-Non Commercial-Share Alike 4.0 License, which allows others to remix, tweak and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

©2025 Asian Pacific Journal of Reproduction Produced by Wolters Kluwer- Medknow.

How to cite this article: Li JR, Zhu SL, Ye NW, Shi FX. Protective effect and mechanism of alpha-lipoic acid on lead-induced Sertoli cell damage in rooster testes. *Asian Pac J Reprod* 2025; 14(4): 179-188.

Article history: Received: 6 December 2024; Revision: 18 March 2025; Accepted: 16 May 2025; Available online: 26 August 2025

oxidative stress in chicken testes, leading to autophagy and apoptosis dysfunction, damaging the blood-testis barrier (BTB). Alpha-lipoic acid (ALA) alleviates reproductive damage induced by lead[4].

In seminiferous tubules, Sertoli cells, spermatogonia, spermatocytes, spermatids, and spermatozoa are present, with Sertoli cells being the only somatic cells that play a crucial role in the testes. Sertoli cells provide nutrients and factors required for the development of germ cells, and many growth factors they produce, such as glial cell line-derived neurotrophic factor (GDNF), bone morphogenetic proteins (BMPs), and stem cell factor (SCF), have been shown to regulate spermatogonial stem cell self-renewal and differentiation[5]. Sertoli cells also perform the functions of phagocytosing degenerated germ cells, protecting germ cells from autoimmune responses, releasing sperm during spermatogenesis, and regulating hormones[6]. Another critical function of Sertoli cells is the formation of the BTB, which provides a stable microenvironment for germ cells and protects developing germ cells by preventing the entry of toxic substances into the seminiferous tubules[7].

Studies have shown that Sertoli cells and the BTB they form are targets for many environmental pollutants in the male reproductive system[8]. Damage to Sertoli cells caused by pollutants leads to the disruption of their secretion functions and the impairment of BTB integrity and permeability, disturbing spermatogenesis and eventually resulting in male infertility[9,10]. Sertoli cells are a good model for evaluating *in vitro* and *in vivo* reproductive toxicity in the male reproductive system and are essential for revealing the reproductive toxicity mechanisms of heavy metals[11]. Cadmium exposure to Sertoli cells causes damage, manifesting as an imbalance in the expression of BTB-related proteins (occludin, ZO-1, *N*-cadherin), disrupting tight junctions and permeability barriers[12,13]. Cadmium chloride can disrupt redox balance, mitochondrial activity, and autophagy, triggering endoplasmic reticulum stress and nuclear factor-erythroid 2-related factor 2 (Nrf2) antioxidant responses, indicating a core role of ROS in the cytotoxicity of cadmium in Sertoli cells[14]. However, there are no reports on lead-induced damage to Sertoli cells in the testes and the underlying mechanisms. Therefore, this experiment aims to explore the effects of lead treatment on Sertoli cells in chicken testes and the regulatory mechanism of ALA.

2. Methods

2.1. Reagents and equipment

Collagenase IV, DMEM-F12 medium, 0.25% trypsin-EDTA, fetal bovine serum (FBS), penicillin-streptomycin were purchased from Invitrogen Gibco; ALA from APExBIO; Lead acetate (99% purity) from Shanghai Bailinwei Chemical Technology Co.; Dimethyl

sulfoxide (DMSO), ROS detection kit, CCK8 cell viability kit, DAPI staining solution, Triton X-100 from Shanghai Biotium Biotechnology Co.; 4% paraformaldehyde from Beijing Solabao Technology Co.

Instruments were: biosafety cabinet (Zhejiang Fuxia Medical, China), CO₂ incubator (Thermo Fisher Scientific, USA), T25 culture flasks, and culture plates (Corning, USA).

2.2. Primary Sertoli cell isolation, purification, and culture

Fifteen six-week-old male chickens (600–800 g) were used in this experiment. Sertoli cells from chicken testes ceased proliferation around the 8th week, with peak proliferation occurring between 3–6 weeks. Therefore, many studies selected 6-week-old chickens for Sertoli cell isolation[15,16]. The detailed steps were as follows:

First, the chicken testes were extracted, washed with saline, and soaked in 75% alcohol for 1 min. In a biosafety cabinet, the testes were washed with phosphate buffer saline (PBS), the tunica albuginea was cut open, and the testes tissue was transferred to a Petri dish. After PBS washing, the testes tissue was finely minced, transferred to a 50 mL centrifuge tube, and PBS was added to mix. The mixture was then allowed to stand for 3 min, the supernatant was discarded, 5 volumes of 1 mg/mL collagenase IV were added, and the tissue was digested in a 37 °C water bath for 30 min with manual mixing halfway through. After digestion, the mixture was centrifuged at 377×*g* for 5 min. Subsequently, the supernatant was discarded, 10 volumes of trypsin were added, and the tissue was digested at 37 °C for 20 min with manual mixing halfway through. After trypsin digestion, an equal volume of medium containing 10% FBS was added to terminate digestion, the solution was passed through a 70 μm filter, and undigested tissue chunks were discarded. The filtrate was centrifuged at 168×*g* for 5 min. The supernatant was discarded, medium with 10% FBS was added, and the cells were transferred to a T25 culture flask.

Sertoli cells were purified using differential adhesion and hypotonic treatment. After 4 h of cell seeding, the medium was removed to eliminate non-adherent germ cells and replaced with fresh medium. After 24 h, 20 mM Tris-HCl (pH 7.4) hypotonic solution was added and the cells were treated for 2 min at room temperature, followed by PBS washing and fresh medium addition.

Culture medium consisted of 10% FBS, DMEM-F12 medium, and 1% Penicillin-Streptomycin. Cells were cultured in a 37 °C incubator with 5% CO₂ and observed daily. The medium was changed every two days, and when cells reached approximately 80% confluence, they were digested with trypsin and subcultured at a 1:2 ratio.

2.3. Experimental design

To prepare stock and working solutions of lead and ALA, lead

acetate was dissolved in sterile water to prepare a 100 mM stock solution, and ALA was dissolved in DMSO to prepare a 1 M stock solution, and stored at -20 °C for later use. The stock solutions were diluted in serum-free DMEM-F12 medium to prepare the working solutions of lead and ALA at different concentrations. When adding the working solutions to the cells, the DMSO concentration was kept at 0.055% in all treatment groups including the control group, which is non-toxic to cells (Dhanasekaran, 2019).

Sertoli cells were treated with various concentrations of lead (0, 2.5, 5, 10, 20, 40, 60, and 160 µmol/L) or ALA (0, 12.5, 25, 50, 100, 200, 400, 800 µmol/L) for 24 h. Cell viability was measured to determine the optimal treatment concentrations. The following groups were established: 1) Serum-free DMEM-F12 medium (the control group); 2) Serum-free DMEM-F12 medium + 40 µmol/L lead acetate (the PB group); 3) Serum-free DMEM-F12 medium + 100 µmol/L ALA (the ALA group); 4) Serum-free DMEM-F12 medium + 40 µmol/L lead acetate + 100 µmol/L ALA (the APB group). In the APB group, cells were pretreated with 100 µmol/L ALA for 4 h, then treated with 40 µmol/L lead acetate for 24 h.

To determine the optimal treatment concentrations for subsequent experiments, we first conducted dose-response studies to evaluate the effects of various lead and ALA concentrations on Sertoli cell viability. Treatment with 5 µM or 10 µM lead did not affect Sertoli cell viability. However, as the lead concentration increased (10, 20, 40, 80, and 160 µM), the survival rate of Sertoli cells gradually decreased. Compared to the untreated control group (0 µM), the cell viability after treatment with 10, 20, and 40 µM lead was (89.12±1.51)%, (83.76±1.91)%, and (74.13±2.62)%, respectively, and these differences were statistically significant. At lead concentrations of 80 µM and 160 µM, the cell viability was close to 50%, indicating significant cell death. Therefore, 40 µM lead was selected for further treatments. There were no significant differences in cell viability among the groups treated with different concentrations of ALA, although a decreasing trend in viability was observed at concentrations above 100 µM. Hence, 100 µM ALA was chosen for subsequent treatments.

2.4. Cell viability assay

Sertoli cells were seeded in a 96-well plate (100 µL per well). After adhesion, the cells were treated accordingly. After treatment, the culture medium was removed, and cells were washed with PBS. Then, 100 µL of medium containing 10% CCK8 solution was added to each well, and the plate was incubated for 4 h in the incubator. Absorbance was measured at 450 nm using a microplate reader. Blank and control wells were set up, and cell viability (%) was calculated as:

$$\text{Cell viability (\%)} = (\text{Treatment group} - \text{Blank group}) / (\text{Control group} - \text{Blank group}) \times 100\%$$

2.5. ROS detection in Sertoli cells

The ROS level in Sertoli cells was measured using a ROS detection kit. After treatment, each well was added with 10 µmol/L DCFH-DA fluorescent probe in serum-free medium. After incubation for 30 min in the incubator, images were taken using a confocal microscope at 488/525 nm. ImageJ software (LAS-4000, Fuji Film, Tokyo, Japan) was used to analyze the average fluorescence intensity of ROS in the cells.

2.6. Western immunoblotting

The total proteins were extracted from Sertoli cells using Radio Immunoprecipitation Assay buffer containing phenylmethylsulfonyl fluoride (Beyotime Biotechnology, Shanghai, China). Protein content was quantified using a bicinchoninic acid protein assay kit (P0010, Beyotime Biotechnology, Shanghai, China). For electrophoresis, 20 µg of protein samples were loaded onto 4%–20% (w/v) SDS-PAGE gels and subsequently transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) at 70 V for 1 h. The membranes were then blocked with 5% bovine serum albumin (BSA) for 2 h at room temperature. After blocking, the membranes were incubated overnight at 4 °C with primary antibodies. GAPDH was used as an internal control. The membranes were then washed three times with Tris-Buffered Saline with Tween 20 (TBST) buffer and incubated with secondary antibody for 60 min at room temperature. After washing three times with TBST, the blots were developed using an enhanced chemiluminescence kit (Thermo Scientific, Wilmington, USA). The immunoreactive bands were visualized using a Luminescent Image Analyzer LAS-4000 (Fuji Film, Tokyo, Japan). Protein expression was quantified by measuring band intensities using ImageJ software. The relative expression of target proteins was normalized to GAPDH. Detailed antibody information is provided in Table 1.

Table 1. Antibodies used in Western blotting analysis.

Antibodies	Supplier	Catalog ID	Dilution
ZO-1	Affinity	AF5145	1:4 500
N-cadherin	Affinity	AF5239	1:1 500
OCN	Proteintech	27260-1-AP	1:1 000
Keap 1 70	Proteintech	10503-2-AP	1:2 000
Nrf2	Abcam	Ab62352	1:2 000
Bax	ABclonal	A19684	1:1 500
Bcl2	ABclonal	A19693	1:1 500
PCNA	Abcam	Ab29	1:3 000
GAPDH	Abcam	Ab8245	1:3 000

2.7. Statistical analysis

Data were analyzed using SPSS software (SPSS 20.0, SPSS, Chicago, USA). Statistical differences between two groups were analyzed with two-tailed Student's *t*-test, while comparisons among more than two groups were performed using one-way ANOVA followed by appropriate *post-hoc* tests. Results were expressed as mean±SD, and differences were considered significant when $P<0.05$.

2.8. Ethics statement

Our study was conducted according to the Declaration of Helsinki. The animal experiments were performed in accordance with the Animal Welfare Committee of Nanjing Agricultural University, Nanjing, China (permit number NJAU/20191683-C1; date: September 2019).

3. Results

3.1. Identification of Sertoli cells from chicken testes

Sex-determining region Y-box 9 (SOX9) and vimentin are proteins specifically expressed in Sertoli cells in testicular tissue. Immunofluorescence staining of SOX9 and vimentin was used to identify Sertoli cells. As shown in Figure 1, the green fluorescence of SOX9 and vimentin surrounds the blue fluorescence of the cell nuclei, with high overlap. The results confirm that the isolated and purified chicken Sertoli cells have high purity, suitable for subsequent experimental research.

3.2. Effects of ALA on lead-induced expression of function-related genes in Sertoli cells

To further investigate the effects of lead and ALA on Sertoli cell function, we measured the expression of function-related genes in Sertoli cells. The results, as shown in Figure 2, indicate that in the PB group, the expression levels of growth-related genes *FGF*, *GDNF*, and *BMP4* in Sertoli cells were significantly lower than those in the control group and the ALA group ($P<0.05$), while the expression level of stem cell factor (SCF) was not significantly different ($P>0.05$). ALA treatment significantly restored the expression of *FGF*, *GDNF*, and *BMP4* in Sertoli cells that were reduced by lead exposure ($P<0.05$). However, ALA treatment had no effect on the expression of SCF ($P>0.05$).

3.3. ALA increases expression of BTB-related proteins in lead-induced Sertoli cells

As shown in Figure 3, compared with the control group, the protein

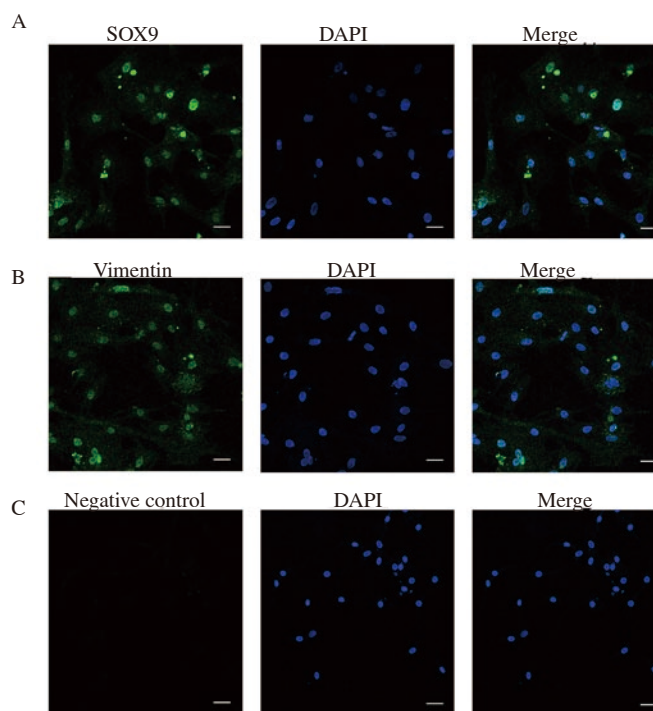


Figure 1. Identification of testicular Sertoli cells in the rooster (magnification 20×). Fluorescence staining of SOX9 (A), vimentin (B), and the negative control (C).

expression levels of ZO-1, *N*-cadherin, and OCLN in the PB group were significantly lower ($P<0.05$). In the ALA group, the protein expression levels of these proteins were not significantly different from the control group ($P>0.05$). Compared with the PB group, the combined treatment of ALA and lead in the APB group significantly increased the protein expression levels of ZO-1, *N*-cadherin, and OCLN ($P<0.05$). There was no significant difference between the control group and the ALA group in the expression of these proteins ($P>0.05$).

The immunofluorescence results of ZO-1 protein in Sertoli cells are shown in Figure 4. Compared with the control group, the fluorescence intensity of ZO-1 in Sertoli cells in the PB group was significantly reduced ($P<0.05$), whereas the fluorescence intensity in the ALA and APB groups showed no significant difference ($P>0.05$). Compared with the PB group, the fluorescence intensity of ZO-1 in the APB group was significantly increased ($P<0.05$).

3.4. Effects of ALA on lead-induced oxidative stress in Sertoli cells

As shown in Figure 5, compared with the control group, the ROS levels in the PB group were significantly increased, and the activities of catalase and superoxide dismutase (SOD) enzymes were significantly decreased ($P<0.05$). ALA treatment significantly reduced the increase in ROS levels induced by lead and enhanced the activity of catalase ($P<0.05$), with no significant effect on SOD activity. There were no significant differences in ROS levels between the control, PB, and APB groups ($P>0.05$). These results suggest that ALA alleviates lead-induced oxidative stress in Sertoli cells.

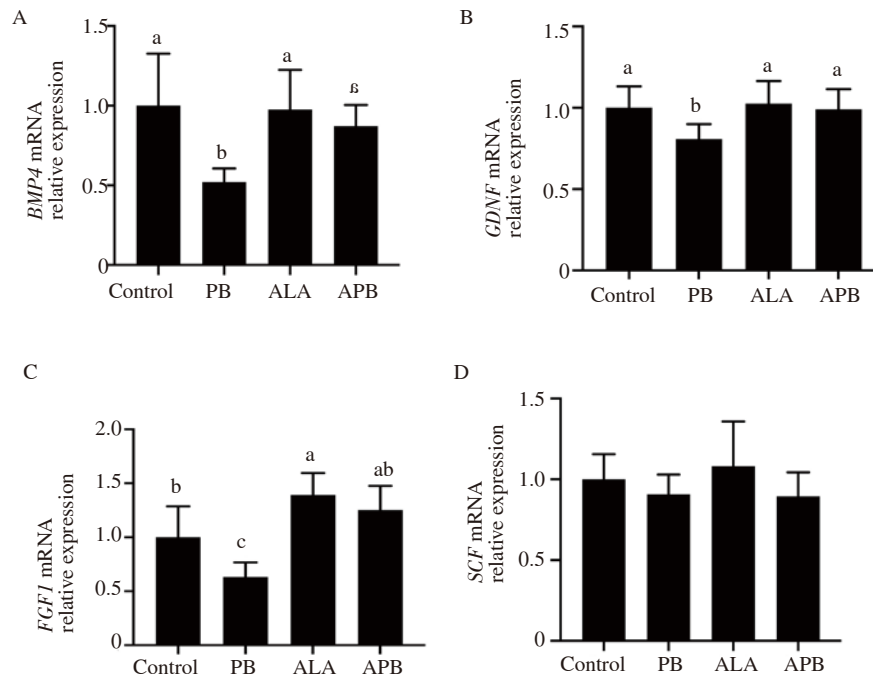


Figure 2. Effects of alpha-lipoic acid (ALA) on Pb-induced mRNA expression levels of function-related gene in rooster testis Sertoli cells. (A) The mRNA expression levels of *BMP4*, (B) *GDNF*, (C) *FGF1*, and (D) *SCF* in the Sertoli cell. Note: Data are represented as means $\pm$ SD; Bars with different letters differ significantly ($P < 0.05$). Stem cell factor (SCF). The control group receives serum-free DMEM-F12 medium; the PB group receives 40 μ mol/L lead acetate (Pb); the ALA group receives 100 μ mol/L alpha-lipoic acid; the APB group receives 40 μ mol/L lead acetate plus 100 μ mol/L alpha-lipoic acid.

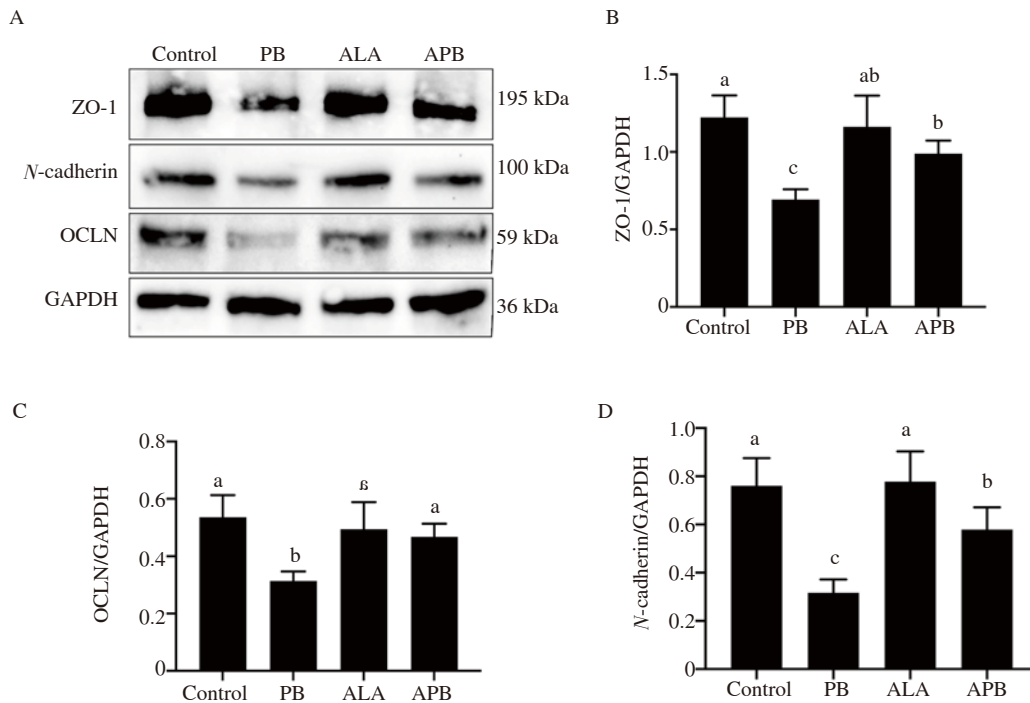


Figure 3. Effects of alpha-lipoic acid (ALA) on Pb-induced protein expression levels of blood-testis barrier (BTB)-related proteins in rooster testis Sertoli cells. (A) Western blot analysis of BTB-related proteins in the Sertoli cell. (B) Statistical analysis of ZO-1/GAPDH, (C) OCLN/GAPDH, and (D) N-cadherin/GAPDH in the Sertoli cell. Note: Data are represented as mean $\pm$ SD; Bars with different letters differ significantly ($P < 0.05$).

3.5. Effects of ALA on the expression of the Nrf2 pathway in lead-induced Sertoli cells

As shown in Figure 6, treatment with lead alone significantly upregulated the expression of Keap1 protein and downregulated the expression of Nrf2 protein and its downstream genes *HO1* and *NQO1*

($P < 0.05$). Compared with the PB group, the APB group showed a significant reduction in Keap1 expression, and a significant increase in the expression of Nrf2 protein and the gene expression levels of *HO1* and *NQO1* ($P < 0.05$). Additionally, there was no effect of lead or ALA treatment on the *GCLC* gene expression levels in Sertoli cells.

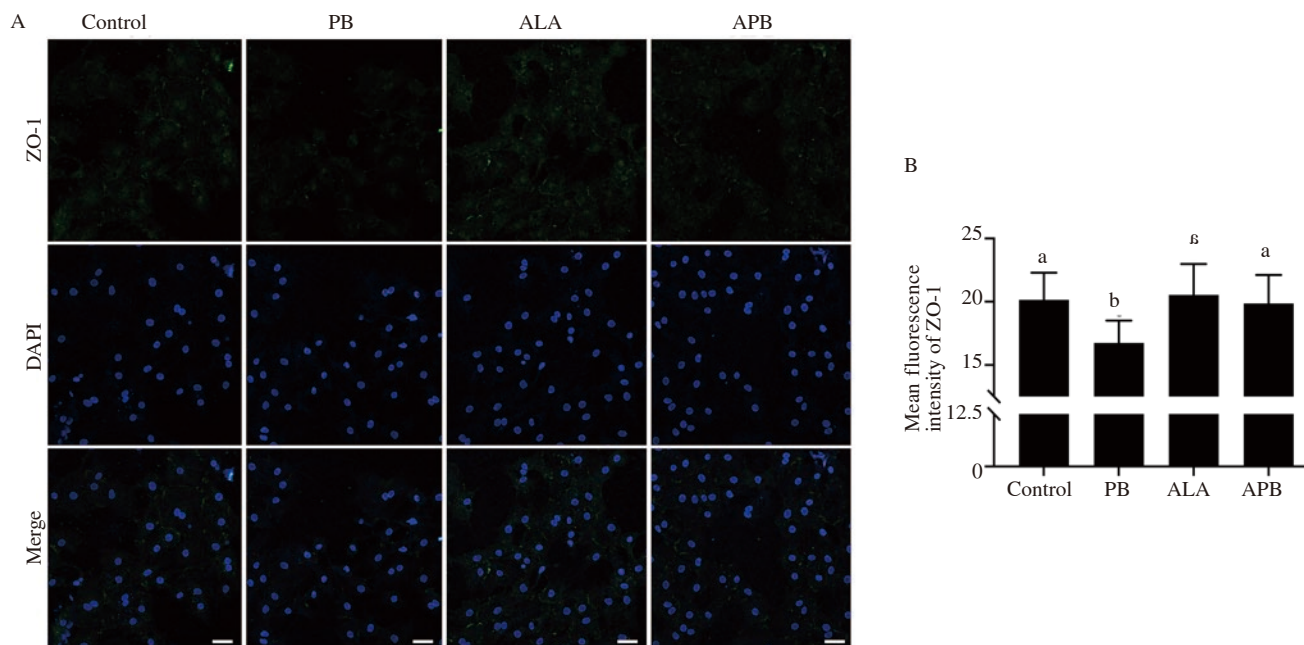


Figure 4. Effects of alpha-lipoic acid (ALA) on Pb-induced protein expression of ZO-1 proteins in rooster testis Sertoli cells. (A) Immunofluorescence analysis of the expression of ZO-1 in the Sertoli cell (magnification 20 $\times$). (B) Statistical analysis of Sertoli cellular ZO-1 protein fluorescence intensity. Note: Data are represented as mean $\pm$ SD; Bars with different letters differ significantly ($P < 0.05$).

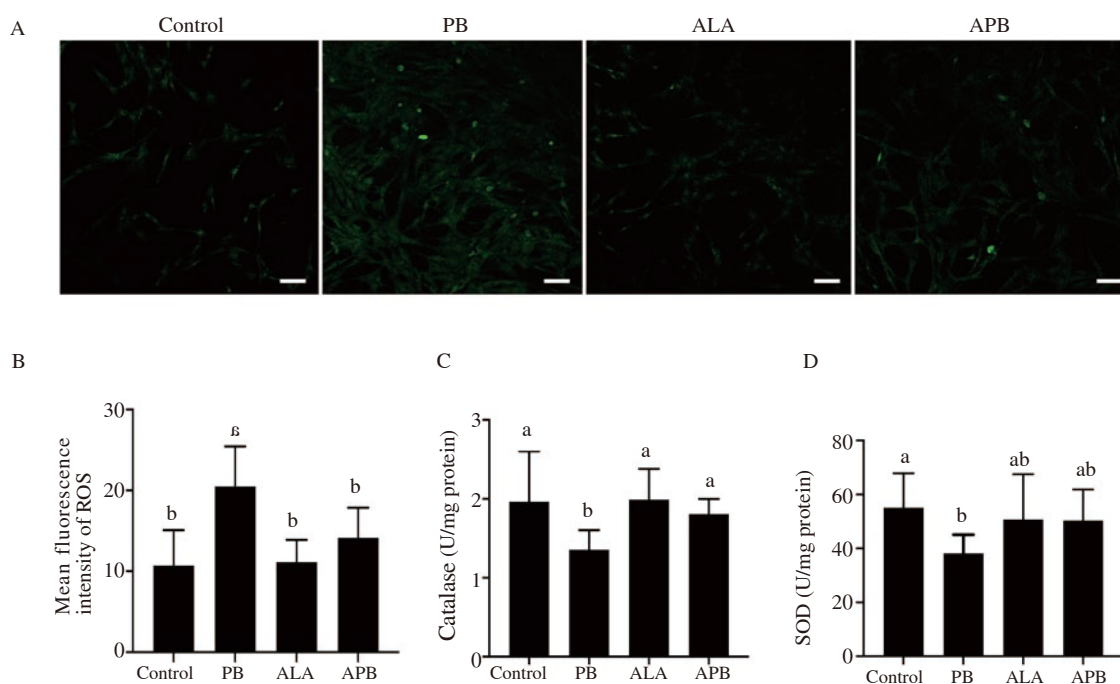


Figure 5. Effect of alpha-lipoic acid (ALA) on Pb-induced oxidative status in Sertoli cells of roosters. (A) Representative images of Sertoli cellular ROS detection (magnification 20 $\times$). (B) Statistical analysis of Sertoli cellular reactive oxygen species (ROS) fluorescence intensity. (C) Catalase activity of Sertoli cells. (D) Superoxide dismutase (SOD) activity of Sertoli cells. Note: Data are represented as mean $\pm$ SD; Bars with different letters differ significantly ($P < 0.05$).

3.6. Effects of ALA on lead-induced apoptosis and proliferation-related protein expression in Sertoli cells

As shown in Figure 7, compared with the control and ALA groups, the expression level of Bcl2 protein in the PB group was significantly reduced, while the expression of Bax protein was

significantly increased ($P < 0.05$). Compared with the PB group, the APB group significantly upregulated the expression of Bcl2 protein and significantly downregulated Bax protein expression ($P < 0.05$). Lead treatment significantly decreased the expression of PCNA protein in Sertoli cells, while combined treatment with ALA and lead significantly increased the expression of PCNA ($P < 0.05$).

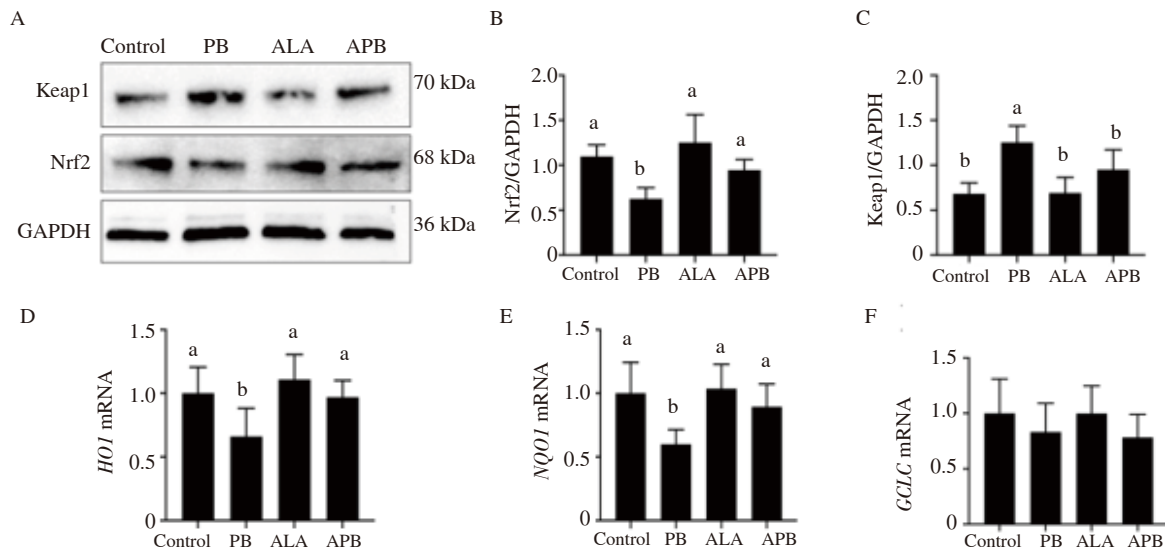


Figure 6. Effects of alpha-lipoic acid (ALA) on Pb-induced protein expression levels of Nrf2 pathway protein and gene in rooster testis Sertoli cells. (A) Western blot analysis of Nrf2 and Keap1 proteins in the Sertoli cell. (B) Statistical analysis of Nrf2/GAPDH and (C) Keap1/GAPDH in the Sertoli cell. (D) The mRNA expression levels of *HO1*, (E) *NQO1*, and (F) *GCLC* in the Sertoli cell. Note: Data are represented as mean ± SD; Bars with different letters differ significantly ($P < 0.05$).

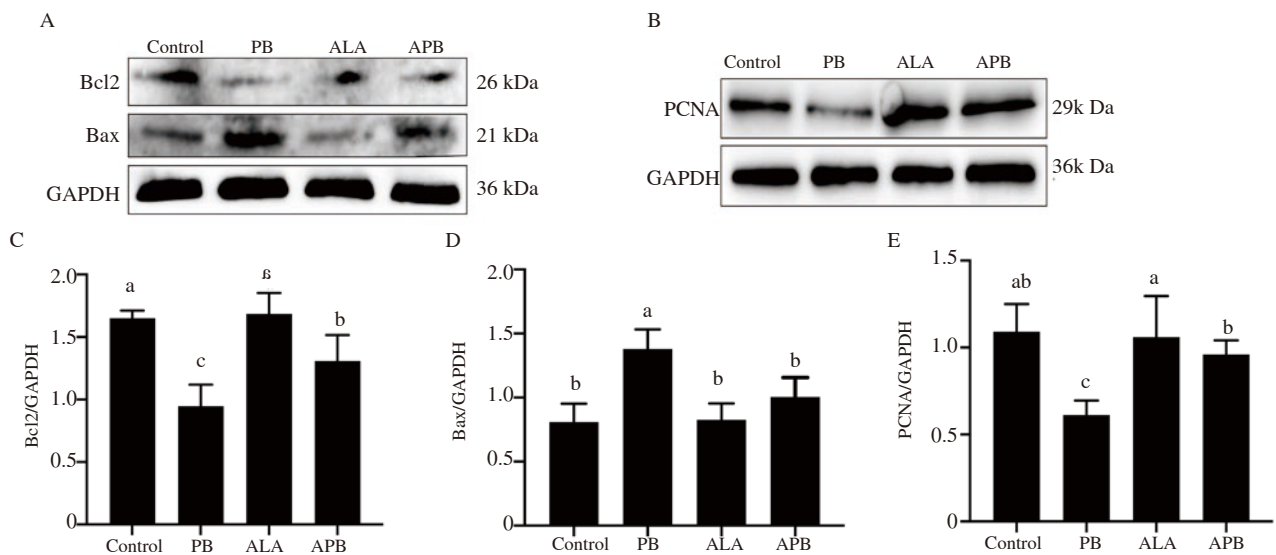


Figure 7. Effects of alpha-lipoic acid (ALA) on Pb-induced protein expression levels of Bcl2, Bax and PCNA in rooster testis Sertoli cells. (A) Western blot analysis of Bcl2 and Bax proteins in the Sertoli cell. (B) Western blot analysis of PCNA in the Sertoli cell. (C) Statistical analysis of Bcl2/GAPDH, (D) Bax/GAPDH, and (E) PCNA/GAPDH in the Sertoli cell. Note: Data are represented as mean ± SD; Bars with different letters differ significantly ($P < 0.05$).

These results indicate that lead treatment promotes apoptosis-related proteins and suppresses proliferation-related proteins in Sertoli cells, while ALA inhibits these changes induced by lead.

4. Discussion

Sertoli cells are involved in regulating spermatogenesis and testicular function, establishing the BTB, preventing the invasion of harmful factors, and ensuring normal sperm production. They play

a vital role in male reproduction[17]. Lead, as a widely distributed environmental pollutant, can accumulate in various organs through water, food, and air, leading to male reproductive damage[18]. The studies in previous studies of this experiment show that lead can damage the BTB and cause testicular damage in chickens. Primary Sertoli cells have been considered a valuable *in vitro* system for studying the effects of heavy metals and toxic substances. In this experiment, Sertoli cells were isolated from 6-week-old chickens using collagenase and trypsin digestion methods, followed by differential adhesion and hypotonic treatment to purify the Sertoli

cells. SOX9 and vimentin are two marker proteins for Sertoli cells[19], and the immunofluorescence results confirm that the isolated and purified Sertoli cells have high purity and are suitable for subsequent experiments.

In the cell viability assay, we found that cell viability decreased with increasing lead concentration. After 24 h of treatment with 40 μ M lead acetate, cell viability was approximately 70%. Yu *et al*[20] demonstrated that when the concentration of lead acetate treatment exceeded 10 μ M, the viability of mouse Sertoli cells significantly decreased. In contrast, 100 μ M ALA significantly increased the survival rate of Sertoli cells after lead treatment. Regulatory factors expressed in Sertoli cells, including FGF, GDNF, BMPs, and SCF, are crucial for the proper execution of spermatogenesis[21,22]. The qPCR results show that lead treatment disrupts the expression of these regulatory factors in Sertoli cells, while ALA alleviates this imbalance. The specialized junction proteins between adjacent Sertoli cells form the BTB, which is one of the key functions of Sertoli cells[7]. It has been reported that BTB-related proteins are targets for several environmental pollutants, leading to barrier disruption[23–25]. *In vitro* cultured rat Sertoli cells have been shown to suffer from disrupted adhesion protein complexes, including tight junction proteins such as occludin-ZO-1 and the basal ES protein complex *N*-cadherin- β -catenin, when exposed to toxins like cadmium[26], bisphenol A[27], or perfluorooctane sulfonic acid[28]. Studies have shown that heavy metals such as lead and cadmium damage the BTB by reducing the levels of tight junction proteins, leading to Sertoli cell damage[29]. Our study indicates that lead exposure decreases the expression of BTB-related proteins in the testes, while ALA can alleviate lead-induced damage to the BTB[4]. The results of the Sertoli cell treatment experiments confirm this, with ALA enhancing the downregulation of ZO-1, OCLN, and *N*-cadherin proteins caused by lead. Therefore, ALA has a protective effect on lead-induced functional disruption of chicken Sertoli cells.

Oxidative stress is considered a central mechanism of toxicity[30,31]. Oxidative stress occurs when the cell's antioxidant capacity is overwhelmed by excessive ROS production. In some studies, excessive ROS induced by lead has been speculated to be a major toxicological response[32]. ROS levels in cells directly reflect the oxidative stress level, and antioxidant enzymes such as catalase, GPx, and SOD form an antioxidant system that can scavenge free radicals and reduce their harmful effects on cells[33]. In this experiment, we found that lead treatment significantly increased intracellular ROS levels and decreased the activities of SOD and catalase enzymes, indicating that lead disrupts the antioxidant defense enzyme system, leading to oxidative stress in Sertoli cells. Similarly, Huang *et al*[3] showed that excessive lead is absorbed by testicular interstitial cells, depleting the cells' antioxidants and producing excess ROS, triggering oxidative stress. It has been found that ALA can enhance the antioxidant system and reduce the accumulation of ROS[34]. Consistent with our results, ALA treatment increased antioxidant enzyme activity and suppressed

ROS production in Sertoli cells, which is related to its ability to scavenge free radicals. Under oxidative stress, Nrf2 dissociates from Keap1 and binds to antioxidant response element (ARE), activating downstream target genes and regulating antioxidant genes such as *HO-1*, *NQO1*, and *GCLC*[35]. ALA has been shown to activate the Nrf2 signaling pathway to enhance antioxidant capacity and protect cells from oxidative damage[36]. Our *in vivo* studies also indicate that ALA enhances Nrf2 expression in chicken testes. After ALA treatment, the Nrf2 protein and the mRNA expression levels of downstream genes *HO-1*, *NQO1*, and *GCLC* in Sertoli cells significantly increased, indicating that Nrf2 mediates the protective effect of ALA against lead-induced Sertoli cell damage.

Autophagy plays a crucial role in maintaining normal cellular homeostasis. At physiological levels, autophagy can maintain cell homeostasis, but excessive autophagy can lead to widespread degradation of cellular components, ultimately resulting in cell death. ROS is an important regulator of autophagy and can induce the formation of autophagosomes. Corsetti *et al*[37] showed that lead induces oxidative stress, causing autophagy in mouse spleen cells. Lead treatment of chicken germ cells induces oxidative stress, further triggering defense responses, including heat shock and autophagy, which ultimately lead to cell damage and reproductive dysfunction. Heavy metal toxins induce autophagy by inhibiting the PI3K/Akt/mTOR signaling pathway[38,39]. Zhou *et al*[40] showed that ALA inhibits 6-OHDA-induced autophagy, thus reducing cell damage. Furthermore, we found that lead treatment reduced the expression of Bcl2 protein and increased Bax protein expression in Sertoli cells, suggesting that lead induces apoptosis, whereas ALA inhibits apoptosis. This result is consistent with our findings in chicken testes. Bcl2, also known as an anti-apoptotic gene, is an important protein that regulates apoptosis and autophagy[41,42]. Previous study found that ALA inhibits induced cell apoptosis by reducing ROS levels and enhancing Bcl2 protein expression[43]. Although our study shows that ALA can mitigate lead-induced autophagy and apoptosis, the exact mechanism and the interaction between autophagy and apoptosis need further investigation.

This study is limited by using only chicken Sertoli cells in an *in vitro* system that cannot replicate the complex testicular microenvironment with its multiple cell interactions. Additionally, the study primarily examined oxidative stress mechanisms without exploring endocrine, inflammatory or epigenetic pathways. Future research should include mammalian models for better translational relevance, investigate optimal ALA dosing and timing, examine long-term reproductive outcomes, explore combination therapies with other protective agents, and develop targeted delivery systems to enhance testicular concentration of ALA. Clinical studies with comprehensive fertility assessments would ultimately be necessary to validate ALA as a potential intervention for lead-induced reproductive toxicity in humans.

In conclusion, lead treatment reduces Sertoli cell viability, induces oxidative stress, and triggers autophagy and apoptosis. ALA

enhances the antioxidant capacity of Sertoli cells by activating the Nrf2 pathway, inhibits autophagy and apoptosis, and alleviates lead-induced Sertoli cell damage.

Conflict of interest statement

The authors declare no competing interests.

Funding

This study was funded by Zhejiang Provincial Natural Science Foundation of China under Grant No. LTGN24C170001, Zhejiang Province Vocational Education Teaching Reform Project jg20230038, Zhejiang Province Education Science Planning Project 2024JCD034, Jinhua Science and Technology Plan Project 2024-2-002.

Authors' contributions

Junrong Li and Shanli Zhu contributed to the conceptualization of this study. Shanli Zhu developed the methodology and conducted the validation and formal analysis. Nanwei Ye handled the software analysis, investigation, resources, and visualization. Junrong Li and Shanli Zhu were responsible for data curation. Shanli Zhu prepared the original draft of the manuscript. Junrong Li and Fangxiong Shi conducted the writing review and editing. Shanli Zhu provided supervision for the research. Fangxiong Shi managed the project administration. Junrong Li and Fangxiong Shi acquired the funding for this research. All authors have read and agreed to the published version of the manuscript.

Publisher's Note

The Publisher of the *Journal* remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

- [1] Zheng X, Guo C, Lv Z, Jiang H, Li S, Yu L, et al. From animal to cell model: Pyroptosis targeted-fibrosis is a novel mechanism of lead-induced testicular toxicity. *Food Chem Toxicol* 2023; **178**: 113886.
- [2] Lopes ACBA, Peixe TS, Mesas AE, Paoliello MM. Lead exposure and oxidative stress: A systematic review. *Rev Environ Contam Toxicol* 2016; **236**: 193-238.
- [3] Huang H, Wang M, Hou L, Lin X, Pan S, Zheng P, et al. A potential mechanism associated with lead-induced spermatogonia and Leydig cell toxicity and mitigative effect of selenium in chicken. *Ecotoxicol Environ Saf* 2021; **209**: 111671.
- [4] Ye N, Lv Z, Dai H, Huang Z, Shi F. Dietary alpha-lipoic acid supplementation improves spermatogenesis and semen quality via antioxidant and anti-apoptotic effects in aged breeder roosters. *Theriogenology* 2021; **159**: 20-27.
- [5] Huang Y, Li L, An G, Yang X, Cui M, Song X, et al. Single-cell multi-omics sequencing of human spermatogenesis reveals a DNA demethylation event associated with male meiotic recombination. *Nat Cell Biol* 2023; **25**(10): 1520-1534.
- [6] Li L, Lin W, Wang Z, Huang R, Xia H, Li Z, et al. Hormone regulation in testicular development and function. *Int J Mol Sci* 2024; **25**(11): 5805.
- [7] Justin Margret J, Jain SK. The protective role of L-cysteine in the regulation of blood–testis barrier functions—A brief review. *Genes* 2024; **15**(9): 1201.
- [8] Jiang B, Yang D, Peng H. Environmental toxins and reproductive health: Unraveling the effects on Sertoli cells and the blood–testis barrier in animals. *Biol Reprod* 2024; **111**(5): 977-986.
- [9] Mao B, Mruk D, Lian Q, Ge R, Li C, Silvestrini B, et al. Mechanistic insights into PFOS-mediated Sertoli cell injury. *Trends Mol Med* 2018; **24**(9): 781-793.
- [10] Ramos-Treviño J, Bassol-Mayagoitia S, Hernández-Ibarra JA, Ruiz-Flores P, Nava-Hernández MP. Toxic effect of cadmium, lead, and arsenic on the Sertoli cell: Mechanisms of damage involved. *DNA Cell Biol* 2018; **37**(7): 600-608.
- [11] Reis MM, Moreira AC, Sousa M, Mathur PP, Oliveira PF, Alves MG. Sertoli cell as a model in male reproductive toxicology: Advantages and disadvantages. *J Appl Toxicol* 2015; **35**(8): 870-883.
- [12] Maretová E, Mareta M, Legáth J. Toxic effects of cadmium on testis of birds and mammals: A review. *Anim Reprod Sci* 2015; **155**: 1-10.
- [13] Wang Y, Jin C, Yi Y, Hu Y, Han X, Tan Z, et al. Rumen-protected glucose stimulates the secretion of reproductive hormones and the mTOR/AKT signaling pathway in the ovaries of early postpartum. *Sci Rep* 2023; **13**(1): 2940.
- [14] Man Y, Liu Y, Xiong C, Zhang Y, Zhang L. Non-lethal concentrations of CdCl₂ cause marked alternations in cellular stress responses within exposed sertoli cell line. *Toxics* 2023; **11**(2): 167.
- [15] Faure M, Guibert E, Crochet S, Chartrin P, Brillard JP, Collin A, et al. Differential proliferation and metabolic activity of Sertoli cells in the testes of broiler and layer breeder chickens. *Poult Sci* 2017; **96**(7): 2459-2470.
- [16] Guibert E, Brière S, Pelletier R, Brillard JP, Froment P. Characterization of chicken Sertoli cells *in vitro*. *Poult Sci* 2011; **90**(6): 1276-1286.
- [17] Ma Y, Yu X, Liu YF, Song B, Sun Z, Zhao S. Immunoregulation and male reproductive function: Impacts and mechanistic insights into inflammation. *Andrology* 2024. doi: 10.1111/andr.13772.
- [18] Kahalerras L, Otmani I, Abdennour C. Wild garlic *Allium triquetrum* L. alleviates lead acetate-induced testicular injuries in rats. *Biol Trace Elem Res* 2022; **200**(5): 2205-2222.
- [19] You X, Chen Q, Yuan D, Zhang C, Zhao H. Common markers of testicular Sertoli cells. *Expert Rev Mol Diagn* 2021; **21**(6): 613-626.
- [20] Yu J, Sun J, Fan Y, Su J, Xie J, Wu Y, et al. Exposure to Pb and Cd alters

- MCT4/CD147 expression and MCT4/CD147-dependent lactate transport in mice Sertoli cells cultured *in vitro*. *Toxicol In Vitro* 2019; **56**: 30–40.
- [21] Jan SZ, Hamer G, Repping S, de Rooij DG, van Pelt AM, Vormer TL. Molecular control of rodent spermatogenesis. *Biochim Biophys Acta* 2012; **1822**(12): 1838–1850.
- [22] Ye N, Lv Z, Huang Z, Cheng Y, Wei Q, Shi F. Dietary folic acid supplementation improves semen quality and spermatogenesis through altering autophagy and histone methylation in the testis of aged broiler breeder roosters. *Theriogenology* 2022; **181**: 8–15.
- [23] Wu D, Liu H, Liu Y, Wei W, Sun Q, Wen D, et al. Protective effect of alpha-lipoic acid on bisphenol A-induced learning and memory impairment in developing mice: nNOS and keap1/Nrf2 pathway. *Food Chem Toxicol* 2021; **154**: 112307.
- [24] Zhang Y, Wu X, Zhu K, Liu S, Yang Y, Yuan D, et al. Icaritin attenuates perfluorooctane sulfonate-induced testicular toxicity by alleviating Sertoli cell injury and downregulating the p38MAPK/MMP9 pathway. *Food Funct* 2022; **13**(6): 3674–3689.
- [25] Zhou GX, Liu WB, Dai LM, Zhu HL, Xiong YW, Li DX, et al. Environmental cadmium impairs blood-testis barrier *via* activating HRI-responsive mitochondrial stress in mice. *Sci Total Environ* 2022; **810**: 152247.
- [26] Siu ER, Wong EW, Mruk DD, Sze K, Porto CS, Cheng CY. An occludin-focal adhesion kinase protein complex at the blood-testis barrier: A study using the cadmium model. *Endocrinology* 2009; **150**(7): 3336–3344.
- [27] Rifa RA, Lavado R. Unveiling the next generation of bisphenol analogs and their impact on human health using *in vitro* methods. *Emerg Contamin* 2024; **10**(2): 100296.
- [28] Wan HT, Mruk DD, Wong CK, Cheng CY. Perfluorooctanesulfonate (PFOS) perturbs male rat Sertoli cell blood-testis barrier function by affecting F-actin organization *via* p-FAK-Tyr407: An *in vitro* study. *Endocrinology* 2014; **155**(1): 249–262.
- [29] Ramos-Trevino J, Bassol-Mayagoitia S, Ruiz-Flores P, Espino-Silva PK, Saucedo-Cárdenas O, Villa-Cedillo SA, et al. *In vitro* evaluation of damage by heavy metals in tight and gap junctions of sertoli cells. *DNA Cell Biol* 2017; **36**(10): 829–836.
- [30] Chen D, Ning F, Zhang J, Tang Y, Teng X. NF- κ B pathway took part in the development of apoptosis mediated by miR-15a and oxidative stress *via* mitochondrial pathway in ammonia-treated chicken splenic lymphocytes. *Sci Total Environ* 2020; **729**: 139017.
- [31] Liu Q, Wang W, Zhang Y, Cui Y, Xu S, Li S. Bisphenol A regulates cytochrome P450 1B1 through miR-27b-3p and induces carp lymphocyte oxidative stress leading to apoptosis. *Fish Shellfish Immunol* 2020; **102**: 489–498.
- [32] Renu K, Chakraborty R, Myakala H, Koti R, Famurewa AC, Madhyastha H, et al. Molecular mechanism of heavy metals (Lead, Chromium, Arsenic, Mercury, Nickel and Cadmium)-induced hepatotoxicity—A review. *Chemosphere* 2021; **271**: 129735.
- [33] Scarlata E, O'Flaherty C. Antioxidant enzymes and male fertility: Lessons from knockout models. *Antioxid Redox Signal* 2020; **32**(8): 569–580.
- [34] Debeissat C, Avalon M, Huart M, Ducheux P, Rodriguez L, Vlaski-Lafarge M, et al. Alpha lipoic-acid potentiates *ex vivo* expansion of human steady-state peripheral blood hematopoietic primitive cells. *Biomolecules* 2022; **12**(3): 431.
- [35] Chen F, Xiao M, Hu S, Wang M. Keap1-Nrf2 pathway: A key mechanism in the occurrence and development of cancer. *Front Oncol* 2024; **14**: 1381467. doi: 10.3389/fonc.2024.1381467.
- [36] Van Nguyen T, Piao CH, Fan YJ, Shin DU, Kim SY, Song HJ, et al. Anti-allergic rhinitis activity of α -lipoic acid *via* balancing Th17/Treg expression and enhancing Nrf2/HO-1 pathway signaling. *Sci Rep* 2020; **10**(1): 12528.
- [37] Corsetti G, Romano C, Stacchiotti A, Pasini E, Dioguardi FS. Endoplasmic reticulum stress and apoptosis triggered by sub-chronic lead exposure in mice spleen: A histopathological study. *Biol Trace Elem Res* 2017; **178**: 86–97.
- [38] Guo M, Wang Y, Zhao H, Wang D, Yin K, Liu Y, et al. Zinc antagonizes common carp (*Cyprinus carpio*) intestinal arsenic poisoning through PI3K/AKT/mTOR signaling cascade and MAPK pathway. *Aquat Toxicol* 2021; **240**: 105986.
- [39] Wang Y, Zhao H, Shao Y, Liu J, Li J, Luo L, et al. Copper or/and arsenic induces autophagy by oxidative stress-related PI3K/AKT/mTOR pathways and cascaded mitochondrial fission in chicken skeletal muscle. *J Inorg Biochem* 2018; **188**: 1–8.
- [40] Zhou L, Cheng Y. Alpha-lipoic acid alleviated 6-OHDA-induced cell damage by inhibiting AMPK/mTOR mediated autophagy. *Neuropharmacology* 2019; **155**: 98–103.
- [41] Green DR, Llamby F. Cell death signaling. *Cold Spring Harb Perspect Biol* 2015; **7**(12): a006080.
- [42] Saddam M, Paul SK, Habib MA, Fahim MA, Mimi A, Islam S, et al. Emerging biomarkers and potential therapeutics of the BCL-2 protein family: the apoptotic and anti-apoptotic context. *Egypt J Med Hum Genet* 2024; **25**(1): 12.
- [43] Vafae F, Derakhshani M, Ghasemzadeh Rahbardo M, Hosseinzadeh H. Alpha-lipoic acid, as an effective agent against toxic elements: A review. *Naunyn-Schmiedeberg's Arch Pharmacol* 2024: 1–28.