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RESEARCH ARTICLE

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Ameliorative effects of zinc and vitamin E against phthalates-induced reproductive toxicity in male rats

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Abstract

Objective: Phthalates (PEs) could cause reproductive harm to males. A mixture of three widely used PEs (MPEs) was used to investigate the ameliorative effects of zinc (Zn) and vitamin E (VE) against male reproductive toxicity.

Methods: Fifty male SD rats were randomly divided into five groups ($n = 10$). Rats in MPEs group were orally treated with 160 mg/kg/d MPEs, while rats in MPEs combined Zn and/or VE groups were treated with 160 mg/kg/d MPEs plus 25 mg/kg/d Zn and/or 25 mg/kg/d VE. After intervention for 70 days, it's was measured of male reproductive organs' weight, histopathological observation of sperms and testes, serum hormones, PIWI proteins and steroidogenic proteins.

Results: Compared with control, anogenital distance, testes weight, epididymides weight, and sex hormones were significantly decreased, while the sperm malformation rate was markedly increased in MPEs group ($p < .05$); the testicular tissues were injured in MPEs group with disordered and decreased spermatids, and arrested spermatogenesis. PIWIL1, PIWIL2, StAR, CYP11A1 and CYP19A1 were down-regulated in MPEs group ($p < .05$). However, the alterations of these parameters were restored in MPEs combined Zn and/or VE groups ($p < .05$).

Conclusion: Zn and/or VE improved steroid hormone metabolism, and inhibited MPEs' male reproductive toxicity.

KEYWORDS

male reproductive toxicity, phthalates, steroidogenic proteins, vitamin E, zinc

1 | INTRODUCTION

Human infertility has developed into a serious social problem all over the world, and malefactor contributes 20%–50% of the infertility cases in humans.[1,2] Semen quality affects male infertility. As reported, men's semen quality parameters were negative correlative with the exposure level of phthalates (PEs).[3–5]

PEs are a family of ubiquitous of synthetic chemicals, and used as plasticizers to increase the flexibility and durability of various products, such as food packaging materials, personal care products, medical devices, toys, etc. Human beings are widely exposed to PEs due to their ubiquitous usage. As reported, PEs and their metabolites were found in human tissues, such as semen, blood, amniotic fluid, breast milk, urine, hair and nails.[6–10] The exposure level of PEs was 23–159 μg/kg/d of the people in China, which might induce health risks.[11]

PEs are recognized environmental endocrine disruptors, and they could harm the reproductive function of male animals. It's reported that dietary exposure to butyl benzyl phthalate (BBP, 750 mg/kg/d) reduced anogenital distance, delayed acquisition of puberty, caused

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retention of nipples and areolae, and malformation of male reproductive system in the male offspring rats.[12] Rats were orally exposed to dibutyl phthalate (DBP, 750 mg/kg/d) in pregnancy induced focal dysgenetic areas in fetal testes.[13] Prenatal exposure to 900 mg/kg/d DBP shortened anogenital distance, caused hypospadias and cryptorchidism in male offsprings in rats.[14] DBP exposure at 500 mg/kg by injection intraperitoneally caused interruption of spermatogenesis, steroidogenesis and fertility in rats.[15] Prenatal exposure to 500 mg/kg/d di(2-ethylhexyl) phthalate (DEHP) also shortened anogenital distance, decreased testes weight, reduced sperm count, and caused cryptorchidism in male offsprings in rats.[16] Oral exposure to DEHP at low doses (30–810 μg/kg/d, 4 weeks) caused prostatic hyperplasia in rats.[17] Exposure to the mixture of BBP, DBP and DEHP at 900 mg/kg/d for 30 days decreased testes weight, serum hormones, and damaged testicular tissues.[18] Exposure to the mixture of six PEs at 160 mg/kg/d for 105 days also decreased serum hormones, changed testicular histological structures and altered testicular steroidogenic proteins.[19,20]

Reproductive function is closely related to the levels of dietary nutrients zinc and vitamin E in the body. As reported, varicocele was inversely correlated with seminal zinc concentration.[21] Case–control studies showed that vitamin E was frequently used to prevent repeated or threatened abortion[22]; sufficient dietary vitamin E could also prevent preterm birth.[23] Animal studies showed that zinc (25 mg/kg/d) could restore chlorpyrifos induced reproductive injury in male rats.[24] 0.5 mg/kg/d zinc sulphate combined 100 mg/kg/d N-acetylcysteine could inhibit DEHP (750 mg/kg/d, 3 weeks) induced testicular oxidative damage in rats.[25] Wang et al.[26] reported that 200 mg/kg/d vitamin E could inhibit 500 mg/kg/d DEHP gestational exposure induced testicular toxicity in the offspring rats. 50 mg/kg/d zinc sulphate combined 15 mg/kg/d vitamin E could alleviate male reproductive injuries caused by 50 mg/kg/d aluminum sulphate in rats.[27] Our previous study reported that 5.25 mg/kg/d zinc supplement ameliorated reproductive injuries induced by PEs in male rats.[28] Zinc combined vitamin E might exert better protective effect on PEs' male reproductive toxicity, and the related studies could offer further insights into reducing health risks induced by PEs exposure.

This study investigated the inhibitive effects of Zn and VE on male reproductive toxicity induced by the mixture of DBP, BBP and DEHP (MPEs), and explored the possible mechanism. Male rats were exposed to MPEs for 70 days. The levels of sex hormones, histological detection of sperms and testes, and the expressions of testicular P-element-induced wimpy testis (PIWI) proteins (PIWIL1 and PIWIL2) were performed for evaluation of male reproductive toxicity in rats. The expressions of testicular steroidogenic proteins (CYP11A1, StAR and CYP19A1) were performed for exploring the possible mechanism.

2 | MATERIALS AND METHODS

2.1 | Reagents and instruments

Zinc sulfate heptahydrate (Cat# Z111853), vitamin E (D-α-Tocopherol, Cat# S161351), BBP (Cat# B109815), DBP (Cat# D639764) and DEHP (Cat# D109648) were purchased from Aladdin (Shanghai, China). Commercial ELISA kits of testosterone (Cat# SU-B30387), luteinizing hormone (LH, Cat# SU-B30447) and follicle-stimulating hormone (FSH, Cat# SU-B34438) for rats were purchased from Nanjing Maibo Biotechnology Co. Ltd (Nanjing, China). Primary antibodies: CYP11A1 (Cat# AF6636), CYP19A1 (Cat# AF6231) and β-actin (Cat# AF0003) were purchased from Beyotime (Shanghai, China); StAR (Cat# A22166), PIWIL1 (Cat# A2150) and PIWIL2 (Cat# A6044) were purchased from ABclonal (Wuhan, China). Secondary antibodies (Cat# A0208, A0216), BCA Protein Assay Kit (Cat# P0010) and radioimmunoprecipitation (RIPA) cell lysis buffe (Cat# P0013B) were also purchased from Beyotime (Shanghai, China). Eppendorf 5417R high-speed refrigerated centrifuge was the product of Eppendorf (Hamburg, Germany); IKA® T10 Basic homogenizer was the product of IKA (Staufen, Germany); Electrophoresis apparatus, Transblot apparatus and Chemiluminescence apparatus were the products of Bio-Rad (Hercules, California, USA).

MPEs was composed of BBP, DBP and DEHP at equipotent toxicity according to their reference dose that is, BBP:DBP:DEHP = 10:5:1.[29]

2.2 | Animals and rat feed

Fifty male Sprague–Dawley (SD) rats (SPF grades, about one month old) were purchased from Zhejiang Vital River Laboratory Animal Technology Co., Ltd. (License No.: SCXK (Zhejiang) 2019-0001). All *rats were* housed in a standard SPF barrier environment with controlled *temperature* (21–24°C), humidity (50%–55%), and lighting (12-h light/12-h dark cycle) conditions. The standard rat feed (NTP-2000 feed) was purchased from Shanghai Puluteng Biotechnology Co., Ltd (Cat# P1100(M01)), and the nutrition compositions of NTP-2000 Feed were showed in Table S1.

2.3 | Experimental design and treatment

The fifty male rats were randomly divided into control group, MPEs group, MPEs + Zn group, MPEs + VE group and MPEs + Zn + VE group according to their body weight, with 10 rats in each group. Rats in the MPEs group were orally treated with 160 mg/kg/d MPEs, as shown in Figure 1, rats in MPEs combined Zn and/or VE groups were orally administered with 160 mg/kg/d MPEs plus 25 mg/kg/d Zn and/or VE. Rats in the control group were orally treated with the excipient 0.5% sodium carboxymethyl cellulose. After 70 days of intervention, about 1.0 mL blood of each rat was collected from their caudal vein, and centrifugated at 4500 *g* for 10 min to collect the serum. After fasted overnight (12 h), all rats were euthanized, then dissected, and the testes, epididymides, periepididymal fats and seminal vesicle of each rat were collected and weighed. Relative organ weight = Organ weight (g)/Body weight (g) × 100%. One testis of each rat was fixed in 10% neutral formalin solution for hematoxylin–eosin (H&E) staining. Serum and the other testes were stored in liquid nitrogen for further analyses. The animal experiment was approved and supervised by the Animal Care and Use Committee and the

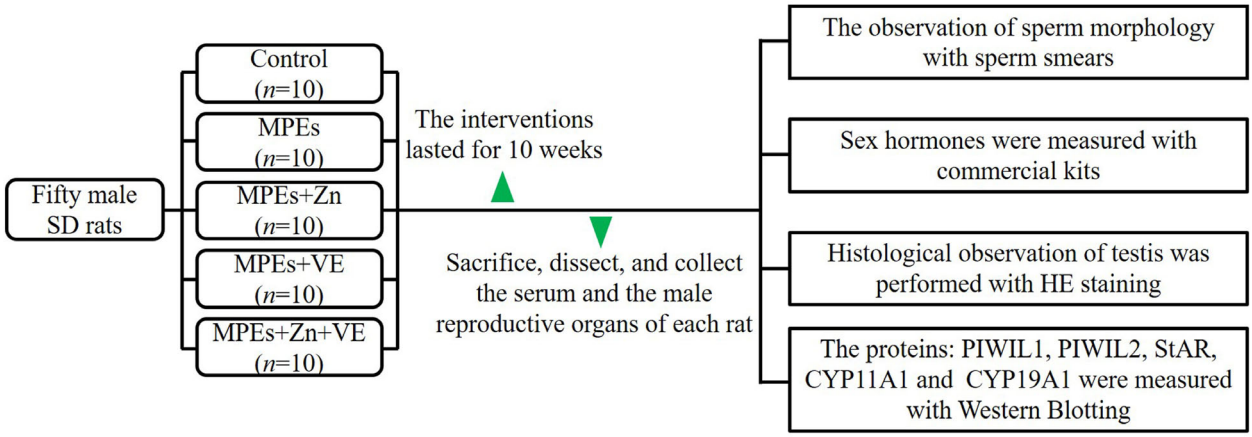

FIGURE 1 A diagram for the experiment. The exposure dose of MPEs was 160 mg/kg/d; The intervention doses of Zn and VE were both 25 mg/kg/d.

Animal Ethics Committee at Wenzhou Medical University (Approval No. xsmq2021-0122).

2.4 | The observation of sperm morphology and abnormal sperm rate

The sperm morphology was evaluated by analysis of sperm smears made from the left cauda epididymis. A longitudinal incision was performed along the cauda epididymis, and placed in 2.5 mL M199 medium at 37°C for 10 min to expel the sperms. Aliquots of 20 μL sperm suspensions were smeared onto glass slides and air-dried. Two sperm smears per sample were performed. The sperm smears were fixed in methanol for 5 min, air-dried, and stained with 1%–2% eosin Y for 60 min followed by microscopic examination. A total of 100 sperms were analyzed on each slide (1000 sperms in each group) for abnormalities of sperms, such as no head, big head, double head, no hook, bent body, amorphous, no tail, tail fold, double tail, etc.

2.5 | The examination of sex hormones

Homogenized testicular tissue (about 100 mg) in cooled 1000 μL RIPA cell lysis buffer was centrifuged at 14000*g* for 30 min at 4°C. Then the supernatants were collected, in which, the protein levels were detected with a BCA Protein Assay Kit. Testicular testosterone, serum testosterone, LH and FSH were detected with the commercial ELISA kits according to their manufacture's instruction.

2.6 | Testicular tissue section observation

The testes tissues were fixed in 10% neutral formalin, embedded in paraffin, and sliced into 5 μm sections which were dewaxed using xylene and rehydrated using ethanol and distilled water. In the next step, the sections were stained with hematoxylin for 15 min, then the color separation was performed with 1% hydrochloride alcohol for about 10 s. After washing with tap water for 15 min, the sections were stained with eosin for 5 s. Following dehydration with ethanol, clearance in xylene, and mounting with neutral gum, the sections were finally viewed under a light microscope (DM4000 B LED, Germany).

2.7 | Western blot assay

The testes tissues were homogenized in 1600 μL pre-cold RIPA assay lysis buffer containing 1 μmol/L phenylmethanesulfonyl fluorid (PMSF), then centrifuged at 14000*g* for 30 min at 4°C. Then the supernatants were collected, and their protein concentrations were measured with BCA Protein Assay Kit. Protein samples were mixed with 5× loading buffer, and boiled for 20 min. The denaturalized protein samples were loaded onto SDS-polyacrylamide gel electrophoresis gels. After electrophoresis, the proteins were transferred to polyvinylidene fluoride membrane. The membranes were cut according to the marker, then blocked in Tris-buffered saline and Tween 20 (TBST) buffer containing 5%–10% non-fat dry milk at room temperature for 2 h, and followed by washing; primary antibodies (1:1000) for the interesting targets were respectively added for incubation at room temperature for 2 h or overnight at 4°C. Then the corresponding secondary antibodies (1:1000) were added for 3 h incubation at room temperature. β-actin was used as an internal control. Bands were visualized and quantized using Bio-Rad Molecular Imager® ChemiDoc™ XRS+ imaging system (Hercules, California, USA).

2.8 | Statistical analysis

The data were expressed as mean $\bar{x}$ ± sd, and analyzed with SPSS 20.0 software (SPSS Inc., USA). Shapiro–Wilk tests were performed for checking data normality. Parametric test (a One-way ANOVA) was employed to analyze the data that followed a normal distribution, while no-parametric test (a Kruskal-Wallis test) were used for the

analysis of data that did not follow a normal distribution. For multiple comparisons of normally distributed data, a One-way ANOVA were performed following by LSD (equal variances assumed) or Dunnett T3 (equal variances not assumed) multiple comparison post-hoc test. A chi-square test was used to compare proportions. $p < .05$ was considered statistically significant. All p values were two tailed.

3 | RESULTS

3.1 | Body weight and anogenital distance

During the experiment, all rats had normal diet and water consumption, and normal appearance and activities. The initial average body weight of rats in each group was about 284 g, and the body weight continuously and rapidly increased in each group during the experiment (Figure 2A,B). At necropsy, compared with control, body weight at necropsy and anogenital distance were significantly decreased in MPEs group (Figure 2B,C, $p < .01$). Whereas, compared with MPEs group, body weight and anogenital distance were significantly increased in MPEs + Zn group, MPEs + VE group and MPEs + Zn + VE group ($p < .05$).

3.2 | The weights of male reproductive organs

Compared with control, it was significantly decreased of testis weight, epididymis weight, periepididymal fat weight in MEPs group (Figure 3A–C, $p < .05$), and the relative periepididymal fat weight in

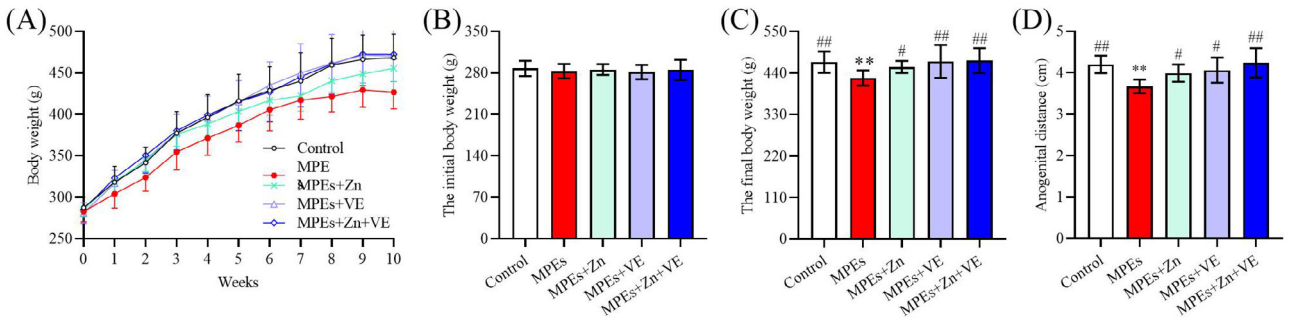

FIGURE 2 The body weight and anogenital distance. (A) the growth curve of the average body weight in each group; (B) the average body weight of the rats in each group at the beginning of the experiment; (C) the average body weights of the rats in each group at necropsy; (D) the average anogenital distance of rats in each group. $^{**}p < .01$ versus control; $^{\#}p < .05$, $^{\#\#}p < .01$ versus MPEs group.

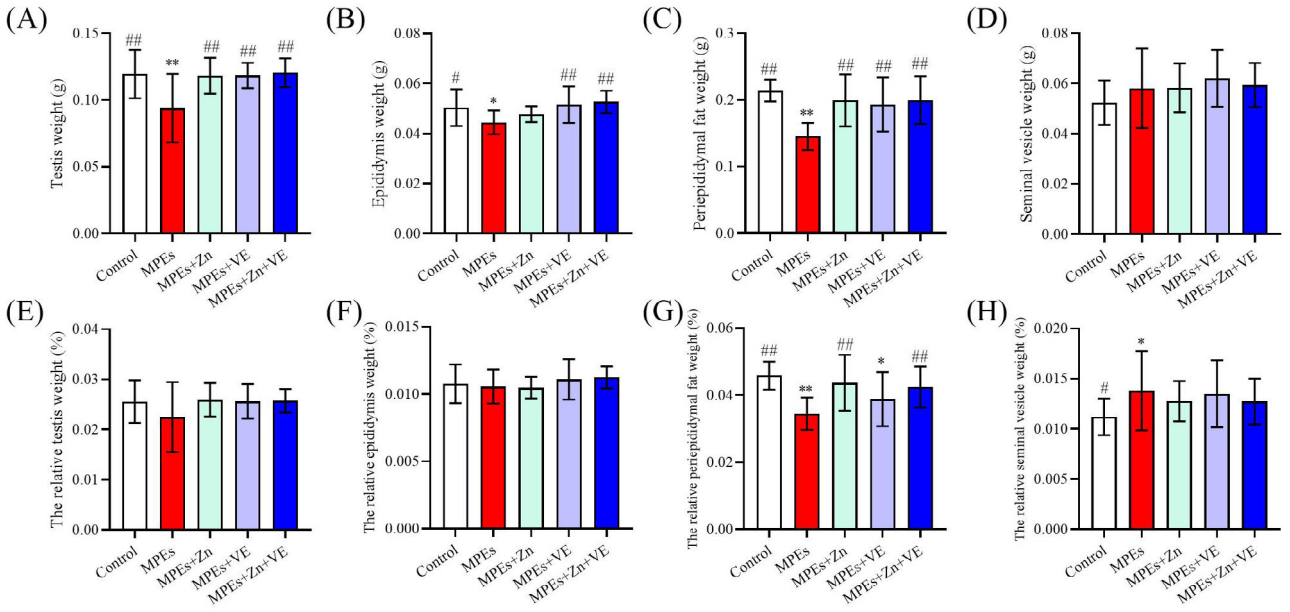

FIGURE 3 The weights of male reproductive organs. (A) testis weight; (B) epididymis weight; (C) periepididymal fat weight; (D) seminal vesicle weight; (E) the relative testis weight; (F) the relative epididymis weight; (G) the relative periepididymal fat weight; (H) the relative seminal vesicle weight. $^{*}p < .05$, $^{**}p < .01$ versus control; $^{\#}p < .05$, $^{\#\#}p < .01$ versus MPEs group.

MEPs group and MPEs + VE group (Figure 3G, $p < .05$); whereas, compared with MPEs group, they were significantly increased in MPEs combined Zn and/or VE groups except epididymis weight in MPEs + Zn group and the relative periepididymal fat weight in MPEs + VE group ($p < .05$). Compared with control, the relative seminal vesicle weight was increased significantly in MEPs group (Figure 3H, $p < .05$). However, there were no significant differences among the five group in seminal vesicle weight, the relative testis weight and the relative epididymis weight ($p > .05$).

3.3 | Sperm morphology and sperm malformation rate

The sperm morphology was analyzed according to the references.[30,31] As shown in Figure 4, the sperms appeared with normal heads and tails in rats in control group (Figure 4A), but plenty of abnormal sperms were found with no head, bent body and no tail in MPEs group (Figure 4B). Abnormal sperms were also found with bent body in MPEs + Zn group (Figure 4C). The vast majority of sperms appeared with a normal head and tail in MPEs + VE group and MPEs + Zn + VE group (Figure 4D,E). As shown in Figure 4F, compared with control, the sperm malformation rates were significantly increased in MPEs treated groups ($p < .01$); whereas, compared with MPEs group, the sperm malformation rates were significantly decreased in MPEs + Zn group, MPEs + VE group and MPEs + Zn + VE group ($p < .01$).

3.4 | The levels of sex hormones

Compared with control, it was significantly decreased of the levels of testicular testosterone, serum testosterone, LH and FSH in MPEs group ($p < .05$). Whereas, as shown in Figure 5, compared with MPEs group, it was significantly increased of the levels of testicular testosterone, serum testosterone and serum LH in MPEs + Zn group, MPEs + VE group and MPEs + Zn + VE group ($p < .05$); serum FSH was also significantly increased in MPEs + Zn + VE group ($p < .05$).

3.5 | Histological observation of testes

Testicular tissue sections were analyzed according the references.[32,33] The spermatids were in the order of spermatogonium, spermatocytes and spermatids from the basal lamina of the seminiferous tubules with distinguishable layers in control group, as shown in Figure 6A, there were numerous sperms in the lumen, and no injuries were found. Testicular histological structure was injured severely in MPEs group, as shown in Figure 6B, the spermatids were arranged in a disordered manner, the number of spermatids was decreased, the spermatogenesis was arrested, and there were few sperms in the lumen. Testicular tissue was moderately injured in MPEs + Zn group, as shown in Figure 6C, the spermatids were arranged orderly, but there were only 2–3 layers of spermatids, the number of spermatids was also reduced. The spermatogenic cells were arranged orderly in MPEs + VE group

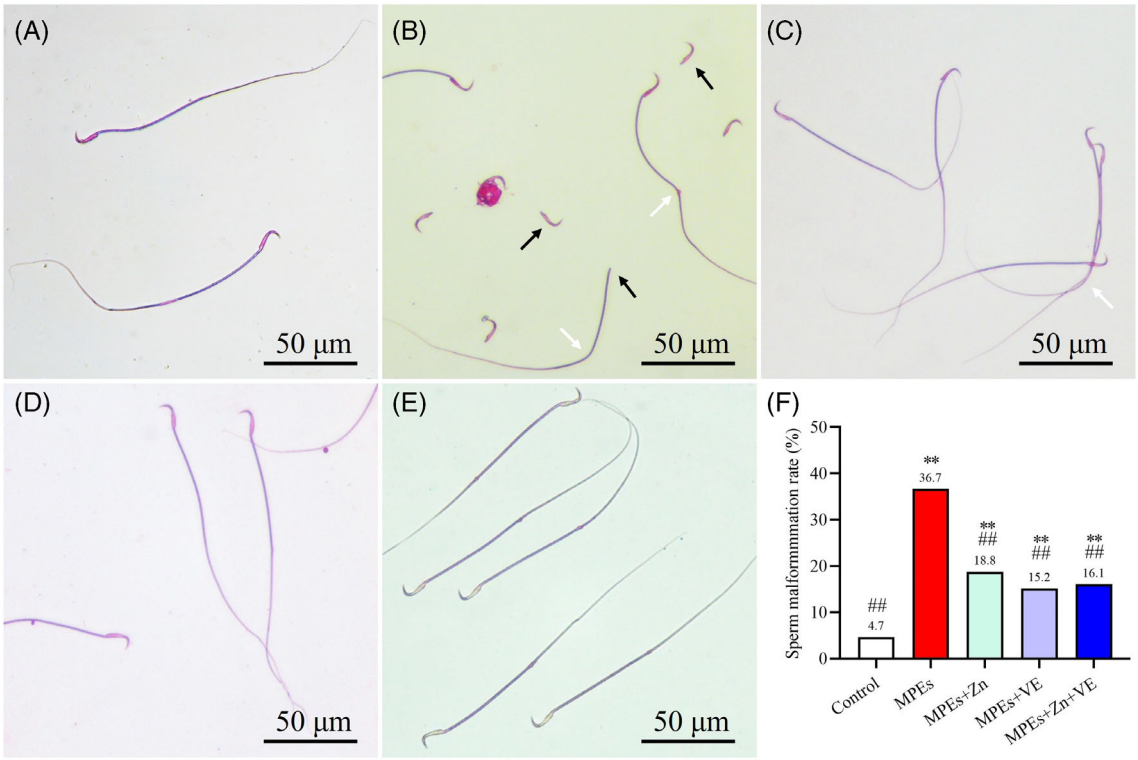

FIGURE 4 Sperm morphology and sperm malformation rate. (A) Control; (B) MPEs group; (C) MPEs + Zn group; (D) MPEs + VE group; (E) MPEs + Zn + VE group; (F) the sperm malformation rate; Black arrows, no head or no tail; White arrows, bent body; $^{**}p < .01$ versus control; $^{\#\#}p < .01$ versus MPEs group. 200×.

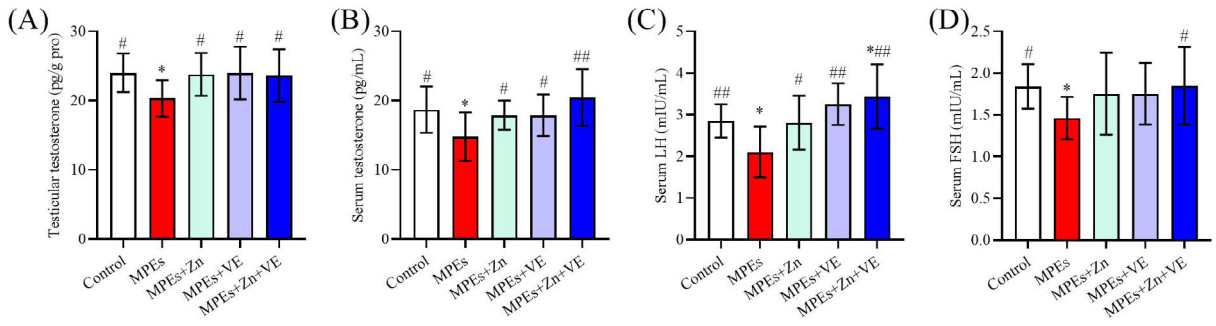

FIGURE 5 Sex hormones levels. (A) testicular testosterone; (B) serum testosterone; (C) serum LH; (D) serum FSH. $^{*}p < .05$ versus control; $^{\#}p < .05$, $^{\#\#}p < .01$ versus MPEs group.

and MPEs + Zn + VE group with distinguishable layers, as shown in Figure 6D,E, there were numerous sperms in the lumen, and there was slight damage.

The testicular tissue injury scoring criteria is shown in Table S2. As shown in Figure 5F, compared with control, the average injury score was significantly increased in MPEs group, MPEs + Zn group and MPEs + VE group ($p < .05$). Whereas, compared with MPEs group, the average injury score was significantly decreased in MPEs + Zn group, MPEs + VE group and MPEs + Zn + VE group ($p < .05$).

3.6 | The expression levels of testicular PIWIL1 and PIWIL2

As shown in Figure 7, compared with control group, the expressions of PIWIL1 and PIWIL2 were down-regulated significantly in MPEs group ($p < .05$). Whereas, compared with MPEs group, the expressions of PIWIL1 and PIWIL2 were up-regulated significantly in MPEs + VE group and MPEs + Zn + VE group ($p < .05$); PIWIL2 was also up-regulated significantly in MPEs + Zn group ($p < .05$).

3.7 | The expression levles of testicular steroidogenic proteins

Compared with control group, as shown in Figure 8, testicular CYP11A1, StAR and CYP19A1 were down-regulated significantly in MPEs group ($p < .05$); Whereas, compared with MPEs group, they were up-regulated significantly in MPEs + VE group and MPEs + Zn + VE group ($p < .05$); CYP17A1 and CYP19A1 were also up-regulated significantly in MPEs + Zn group ($p < .01$).

4 | DISCUSSION

Human beings are inevitably exposing to the mixture of ubiquitous PEs, which may imply health risks. DEHP, DBP and BBP are commonly and largely used PEs. They were identified as priority controlled pollutants and widely detected in human urine, blood and semen.[6,34–36] The mixture of three commonly and largely used PEs: BBP, DBP and DEHP was used in this study to simulate people exposing to the complexes of PEs. The exposure dose of MPEs (160 mg/kg/d) was designed based on the previous reports,[19] while the intervention doses of Zn (25 mg/kg/d) and VE (25 mg/kg/d) were designed based on the previously published studies.[24,27,37] After70 days of continuous intervention, MPEs exposure decreased anogenital distance, testes weight, epididymides weight, periepididymal fat weight and sex hormones, increased sperm malformation rate and altered the histological structures of testicular tissue, which were in line with the previous studies.[19,27,38] These results indicated that MPEs exposure induced male reproductive injuries in rats. On the other hand, these alterations were inhibited in MPEs combined Zn and/or VE groups, indicating that Zn and VE inhibited MPEs' male reproductive toxicity.

A short male anogenital distance was strongly associated with genital malformations at birth and reproductive disorders in adulthood, and anogenital distance was used as an indicator for reproductive toxicity in rodent toxicity studies.[39] Morova et al.[40] reported that gestational exposure to the mixture of diethylhexyl, diisononyl and dibutyl phthalate, each at a dose of 4.5 mg/kg/d, shortened anogenital distance of the male offspring in rats. DBP (1–500 mg/kg/d) also caused reduce of anogenital distance following prepubertal mice exposure to DBP from 4 to 14 days of age.[41] In the present study, the shortened anogenital distance indicated that MPEs exposure caused reproductive injury in rats in MPEs group. Periepididymal fat could protect testes and epididymides from damages, and maintain normal male reproductive function. Both decrease of periepididymal fat weight and the relative periepididymal fat weight also indicated male reproductive injury in rats in MPEs group. The increased sperm malformation rate and the changes of testicular histological structures added evidences to the male reproductive injuries induced by MPEs exposure. In contrast, the supplement of Zn and/or VE inhibited the the alterations of these parameters, implied that Zn and VE could alleviate the male reproductive injuries induced by MPEs in rats. Our results were agreed with the studies of Chemek et al.[42] and Wang et al.[26] Chemek et al.[42] reported that Zn could inhibit the decrease of the relative testis weight and the relative epididymis weight in male offspring rats induced by gestational and lactational exposure to cadmium. Wang et al.[26] argued that VE (200 mg/kg/d) could suppress the

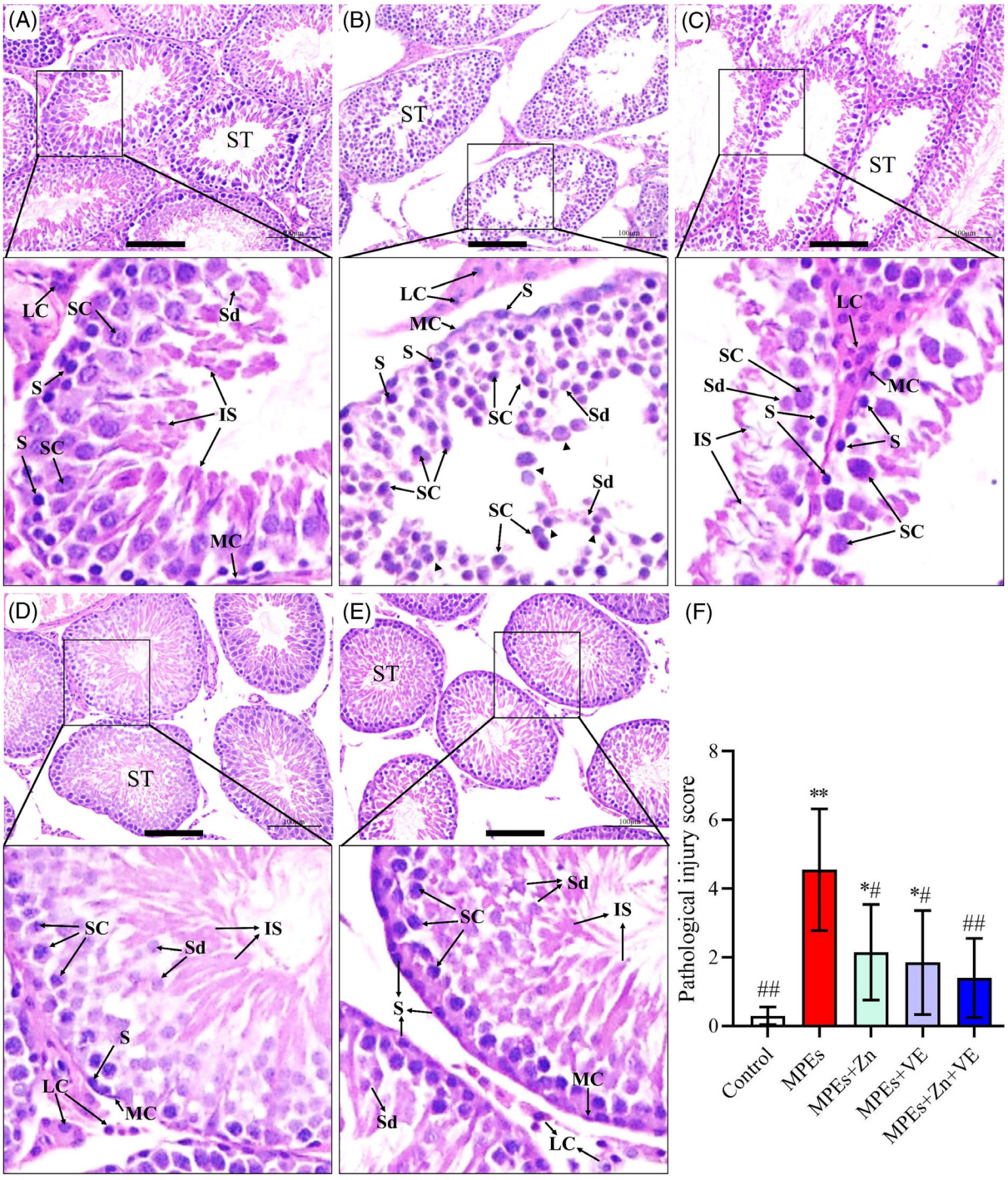

FIGURE 6 Histological observation of testicular tissues. (A) control; (B) MPEs group; (C) MPEs + Zn group; (D) MPEs + VE group; (E) MPEs + Zn + VE group; IS, immature sperm; LC, leydig cells; MC, myoid cells; S, spermatogonia; SC, spermatocytes; Sd, spermatids; ST, seminiferous tubule; ▲, arrested spermatocytes. $^{*}p < .05$, $^{**}p < .01$ versus control; $^{\#}p < .05$, $^{\#\#}p < .01$ versus MPEs group. 200×, bar = 100 μm.

decrease of body weight and testis weight, and injuries of testicular tissue in male offspring rats following gestational expsure to 500 mg/kg/d DEHP.

PIWI proteins, such as PIWIL1, PIWIL2 and PIWIL4, are germline-specifically expressed in mammals' germ cells and they could protect germ cells from the activity of mobile genetic elements, such as transposons, playing a crucial role in germ line maintenance, differentiation and meiosis conserved in mammals.[43–45] PIWIL1 is specifically expressed in germ cells, and loss of PIWIL1 indicated that the germ cells failed to enter the differentiation pathway.[46] PIWIL2 is required for germline stem cell self-renewal, and PIWIL2 deficiency leads to defects in meiosis and germ cell survival.[47] It's reported that male mice knockout of PIWIL1 or PIWIL2 were infertile.[45,48] And they could be used as indicators for male reproductive function.[33,49] In this

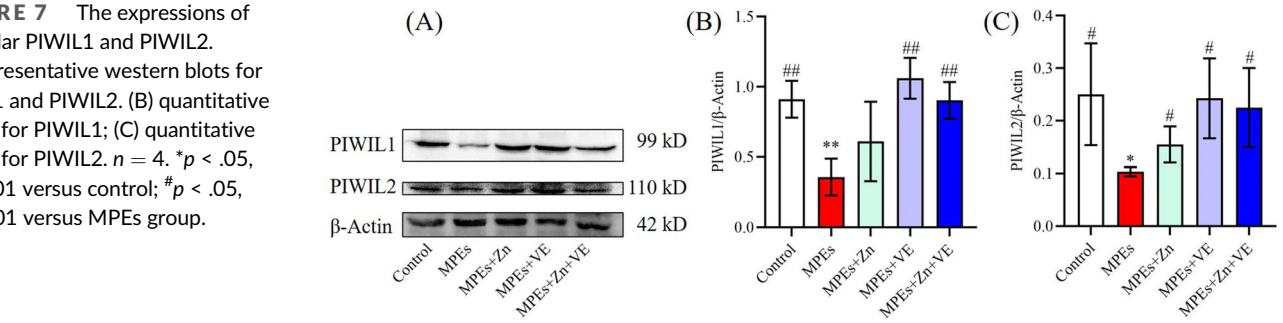

FIGURE 7 The expressions of testicular PIWIL1 and PIWIL2. (A) representative western blots for PIWIL1 and PIWIL2. (B) quantitative results for PIWIL1; (C) quantitative results for PIWIL2. $n = 4$. $^{*}p < .05$, $^{**}p < .01$ versus control; $^{\#}p < .05$, $^{\#\#}p < .01$ versus MPEs group.

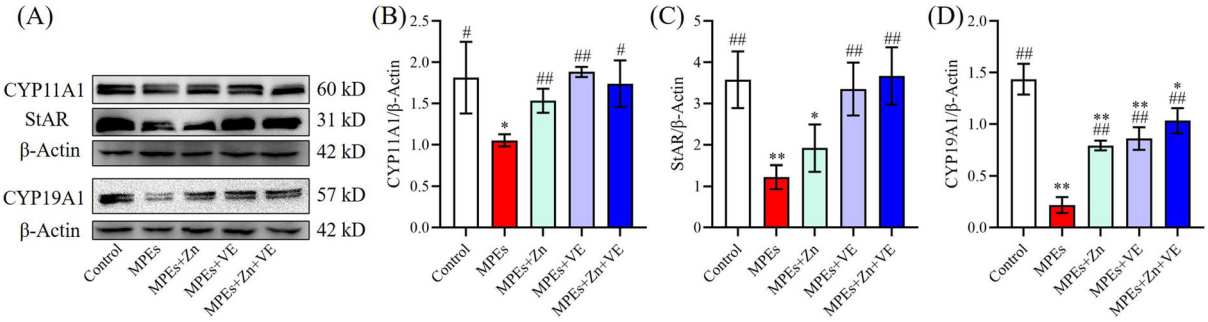

FIGURE 8 The expressions of testicular steroidogenic proteins. (A) representative western blots for steroidogenic proteins. (B) quantitative results for CYP11A1; (C) quantitative results for StAR; (D) quantitative results for CYP19A1. $n = 3$. $^{*}p < .05$, $^{**}p < .01$ versus control; $^{\#}p < .05$, $^{\#\#}p < .01$ versus MPEs group.

study, the down-regulations of testicular PIWIL1 and PIWIL2 in MPEs group were consistent with Pan et al.,[50] who argued that estrogen exposure down-regulated the expression of testicular PIWIL1 and PIWIL2 in mice. Our previous study also showed that it's barely expressed of testicular PIWIL1 and PIWIL2 following the rats expsorue to 900 mg/kg/d MEPs for 30 days.[33] This result added to the evidences that 160 mg/kg/d MPEs caused reproductive injuries in male rats. On the other hand, the down-regulation of testicular PIWIL1 and PIWIL2 were mitigated by Zn and/or VE, further indicating that Zn and/or VE inhibited MPEs' testicular toxicity.

The key male reproductive organ in animals is testes, which perform two crucial functions, that is, spermatogenesis (the production of spermatozoa) and steroidogenesis (the biosynthesis of steroid hormones). In spermatogenesis, testosterone stimulates sperm production in adult males, and maintains male reproductive function. As the principal steroid hormone from the androgenic group, testosterone is biosynthesized in Leydig cells in testis. Testosterone synthesis in the testes is tightly regulated by hypothalamic–pituitary-gonad (HPG) axis, in which, LH and FSH play vital roles. LH binds to the LH receptors, and stimulates testosterone production via HPG axis; FSH promotes spermatogenesis via HPG axis. Previous studies showed that PEs decreased serum level of testosterone in male rats.[28,33,51,52] In the present study, the levels of testicular testosterone and serum testosterone were both decreased in MPEs group, so did the serum levels of LH and FSH, implying that MPEs exposure inhibited the spermatogenesis and steroidogenesis in rats, which was further demonstrated by the result of testicular morphology. On the other hand, supplement of Zn and/or VE increased the levels sex hormones and improved testicular histological structures, and further proved that Zn and/or VE suppressed the MPEs' male reproductive toxicity.

Testosterone is the principal androgen, and maintains normal reproductive function in male mammals. It's reported that more than 90% of testosterone is synthesized by Leydig cells via steroid hormone metabolism.[53] In this pathway, StAR is responsible for the translocation of cholesterol from the cytosol into mitochondria, then the cholesterol is converted into pregnenolone by CYP11A1. Subsequently, a series of enzymes, such as CYP17A1, 3β-HSD and 17β-HSD, catalyze the conversion of pregnenolone to testosterone, which can be catalyzed into dihydrotestosterone by 5α-reductase, and excreted with urine. Testosterone can also be converted into estrogens by CYP19A1. The alteration of these steroidogenic proteins would change the testosterone level, and injure male reproductive functions.

The results of the present study showed that MPEs down-regulated testicular StAR, CYP11A1 and CYP19A1, which were consistent with the results of Gao et al.,[19,20] who argued that exposure to the mixture of six PEs at 160 mg/kg/d for 15 weeks also down-regulated testicular StAR and CYP11A1 in rats, as well as down-regulated testicular CYP17A1 and 17β-HSD. Lv et al.[54] reported that dicyclohexyl phthalate exposure (100- and 1000 mg/kg/d) down-regulated testicular StAR, CYP11A1, CYP17A1, 3β-HSD and 17β-HSD in rats. Pan et al[55] argued that dietary exposure

to 450 mg/kg/d diisobutyl phthalate for 4 weeks also down-regulated testicular StAR, CYP11A1 and 3β-HSD in mice. The results of our study implied that MPEs exposure at 160 mg/kg/d suppressed steroid hormone metabolism, and inhibited the biosynthesis of testosterone, which might be the reason that the levels of testicular testosterone and serum testosterone were decreased in MPEs group. Interestingly, the levels of serum LH and FSH were also decreased in MPEs group, which was in agreement with the study of Lv et al.,[54] who argued that dicyclohexyl phthalate exposure (100- and 1000 mg/kg/d) decreased serum LH and FSH in rats; this result also indicated that MPEs exposure perturbed HPG axis in rats. Ha et al.[38] also argued that DEHP (250-, 500-, 750 mg/kg/d, 30 days) disturbed HPG axis, and decrease serum testosterone, LH and FSH in rats. On the other hand, the down-regulation of StAR, CYP11A1 and CYP19A1 were restored in MPEs combined Zn and/or VE groups, as well as that the decrease of testosterone, LH and FSH were also restored, indicating that Zn and VE promoted steroid hormone metabolism and improved HPG axis system.

5 | CONCLUSION

In summary, our results indicated that MPEs exposure disturbed HPG axis system, suppressed steroid hormone metabolism and caused male reproductive toxicity in rats. Zn and VE could inhibit MPEs' male reproductive toxicity, which may relate to the improved regulation of steroid hormone metabolism and improved HPG axis system.

AUTHOR CONTRIBUTIONS

This work was performed as a collaboration among all authors. Ling-Zi XIA, Hai-Tao GAO and Burong HU were involved in the conception and design of this study. Ling-Zi XIA, Li-Lan LIU, Jun-Zhe YUE, Zhen-Yu LU, Ru-Ya DENG, Xi HE and Can-Can LI performed the experimental work, data analysis and interpretation of the results. Ling-Zi XIA and Li-Lan LIU prepared the first draft of the manuscript. Hai-Tao GAO and Burong HU did a critical revision of the manuscript, and contributed significantly in the revision (suggested by reviewers) of manuscript. Hai-Tao GAO supervised the study.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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