Toxicology and Applied Pharmacology 483 (2024) 116816

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/taap

Long-term exposure to the mixture of phthalates induced male reproductive toxicity in rats and the alleviative effects of quercetin

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ARTICLE INFO

Editor: Lawrence Lash

Keywords:
Phthalates
Male reproductive toxicity
Quercetin
Steroid hormone metabolism

ABSTRACT

Phthalates (PEs), such as di(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP) and butyl benzyl phthalate (BBP) could cause reproductive and developmental toxicities, while human beings are increasingly exposed to them at low-doses. Phytochemical quercetin (Que) is a flavonoid that has estrogenic effect, anti-inflammatory and anti-oxidant effects. This study was conducted to assess the alleviative effect of Que. on male reproductive toxicity induced by the mixture of three commonly used PEs (MPEs) at low-dose in rats, and explore the underlying mechanism. Male rats were treated with MPEs (16 mg/kg/day) and/or Que. (50 mg/kg/d) for 91 days. The results showed that MPEs exposure caused male reproductive injuries, such as decreased serum sex hormones levels, abnormal testicular pathological structure, increased abnormal sperm rate and changed expressions of PIWIL1 and PIWIL2. Furthermore, MPEs also changed the expression of steroidogenic proteins in steroid hormone metabolism, including StAR, CYP11A1, CYP17A1, 17β-HSD, CYP19A1. However, the alterations of these parameters were reversed by Que. MPEs caused male reproductive injuries in rats; Que. inhibited MPEs' male reproductive toxicity, which might relate to the improvement of testosterone biosynthesis.

1. Introduction

Infertility is a highly ubiquitous global health problem, affecting about 12.6% to 17.5% of reproductive-age couples, half of which are caused by male factors, such as sperm quality decline (Agarwal et al., 2021; Cox et al., 2022). Previous studies have revealed that infertility is closely related to environmental factors (Cannarella et al., 2023; Deng et al., 2022; Mesquita et al., 2021).

Phthalates (PEs) are used as plasticizers in consumer products and have become unavoidable in the human routine lifestyle (Arrigo et al., 2023). PEs are regarded as contaminants because they are not chemically bound to product structures and thus can escape into the environment, such as air, water and soil (Kumari and Pulimi, 2023). It's reported that, of all known PEs congeners, di(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP) and butyl benzyl phthalate (BBP) were identified as priority controlled pollutants and widely detected in human urine, blood, and semen (Cui et al., 2022; Kumawat et al., 2022; Li et al., 2022; Yu et al., 2021). Further researches reported that semen quality parameters (sperm concentration, sperm viability and progressive activity) and sex hormones levels were negatively correlated with the levels of PEs' metabolites, suggesting that PEs might lead to infertility (Chang et al., 2022; Wang et al., 2016; You et al., 2015). In parallelly, animal studies also argued that PEs exposure induced male reproductive injuries, such as abnormal sex hormones levels, pathological changes in testes, and reduced sperm production (Conley et al., 2021; Deng et al., 2021; Hua et al., 2023).

As the major male reproductive organs, testes mainly consist of seminiferous tubules and interstitial areas. Spermatogenesis takes place in seminiferous tubules, which contain germ cells and Sertoli cells. Leydig cells reside in the interstitial areas. Testosterone is essential for spermatogenesis and male fertility, and most of them are synthesized by Leydig cells (Liu et al., 2023). Studies showed that PEs and their metabolites could act on Leydig cells, altering the key proteins (StAR, CYP17A1, 17β-HSD and *etc.*) in the steroid hormone metabolism pathway, and resulted in a decrease in testosterone level, which seriously affected male reproductive and sexual functions (Wang et al., 2021a; Yang et al., 2020b). Additionally, PEs could also directly affect the development and function of Leydig cells, leading to a decrease in

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https://doi.org/10.1016/j.taap.2024.116816
Received 15 August 2023; Received in revised form 30 December 2023; Accepted 9 January 2024
Available online 11 January 2024

the weight of testes and accessory reproductive organs, as well as a decrease in testosterone level (Oudir et al., 2018). However, the toxicological studies on PEs mainly focused on single substance, high-dose, acute and short-term animal exposure experiments, which failure to simulate the actual characteristics of human chronic exposure to the mixture of PEs at low-doses (Liu et al., 2023; Taşlıdere et al., 2022). Human beings are inevitably exposing to the mixture of ubiquitous PEs, which may imply health risks, and some protective interventions are advised to be carried out.

Quercetin (Que, $C_{15}H_{10}O_7$), also known as 3,3′,4′,5,7-pentahydroxyflavone, is a naturally occurring flavonoid, and widely distributed in fruits, vegetables and medicinal herbs (Rotimi et al., 2022). The molecular structure of Que. is composed of two benzene rings (A ring and B ring) linked by a heterocyclic pyran ring (C ring). As illustrated in Fig. 1 (IV), the molecular structure of Que. contains a dihydroxy group between the A ring, o-dihydroxy group B, C ring C2, C3 double bond, as well as 4-carbonyl, and the biological activity of Que. is largely attributed to these active groups (Nguyen and Bhattacharya, 2022). Que. has many pharmacological activities, including antioxidation, anti-inflammatory, antibiotic, scavenging free radicals, and improving immunity (Yang et al., 2020a). Studies showed that Que. had positive effect on male reproductive function, such as regulating sex hormone levels, increasing sperm quantity, motility, and viability, restoring histopathological structure (Behairy et al., 2022; Oyewopo et al., 2021; Zhang et al., 2023). It's reported that environmental pollutants exposure induced oxidative stress, disturbed hypothalamic-pituitary-testicular axis and caused the reduction of testicular function; whereas, Que. ameliorated male reproductive toxicity, largely by scavenging ROS, increasing the activities of various antioxidant enzymes and inhibiting the activity of pro-oxidative enzymes (Rotimi et al., 2022). Additionally, previous studies also revealed that Que. restored the alterations of the key proteins in testosterone biosynthesis caused by pesticides, heavy metals, and *etc.* (Abarikwu and Farombi, 2016; Sharma et al., 2018; Ujah et al., 2018). For instance, Que. attenuated cadmium chloride induced male reproductive toxicity in rats, restored the alterations of steroidogenic proteins, such as 3β-HSD and 17β-HSD, and restored testosterone levels (Ujah et al., 2018). Our previous study showed that Que. inhibited male reproductive damage induced by high-dose (900 mg/kg/d) PEs' mixture for 30 days in rats, which might relate to the improved regulation of steroid hormone metabolism (Xia et al., 2023). However, it's more significance in practice of the studies simulating people chronic exposure to the mixture of PEs at low-doses.

Based on the above studies, PEs exposure could disturb steroid hormone metabolism. In this study, male rats were orally treated with the mixture of DEHP, BBP and DBP (MPEs) at low-dose for long-term imitating people chronic exposure to the mixture of PEs, to study the inhibitive effect of Que. on MPEs' male reproductive toxicity and explore the mechanism based on steroid hormone metabolism.

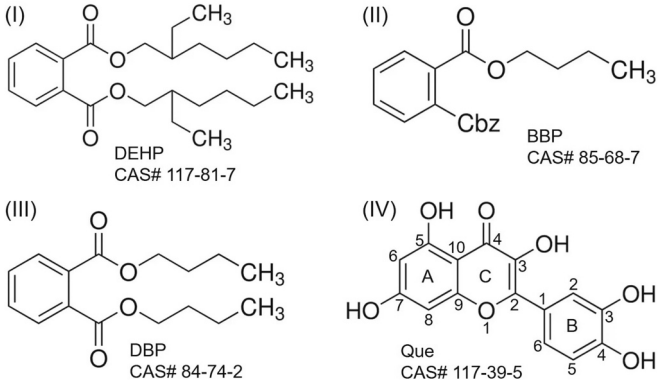

Fig. 1. The molecular structures of DEHP, BBP, DBP and Quercetin (Que).

2. Material and methods

2.1. Materials and animals

DEHP (CAS# 117–81-7), BBP (CAS# 85–68-7), DBP (CAS# 84–74-2) and Que. (CAS# 117–39-5) were purchased from Aladdin (Shanghai, China). Commercial ELISA kits of testosterone, LH, FSH, dehydroepiandrosterone, androstenedione, estrone and dihydrotestosterone for rats were gained from Nanjing Maibo Biotechnology Co. Ltd. (Nanjing, China). The primary antibodies' information was listed in Table 1. BCA Protein Assay Kit, RIPA Cell Lysis Buffer, pre-stained protein molecular weight standards and secondary antibodies were the products of Beyotime (Shanghai, China).

Forty-eight male Sprague-Dawley (SD) rats (SPF grades, weighting about 100 g) were obtained from Zhejiang Vital River Laboratory Animal Technology Co., Ltd. (License No.: SCXK (Zhejiang) 2021–0006). All rats were housed under controlled temperature (21 °C–24 °C), humidity (50%–55%), and lighting (12-h light/12-h dark cycle) conditions.

2.2. Experimental design and treatment

MPEs was the isotoxic mixture of DEHP, DBP and BBP according to their reference dose (US EPA, 2007), that was, DEHP:DBP:BBP = 1:5:10. The forty-eight male SD rats were randomly and evenly divided into control group, Que. group, MPEs group and MPEs+Que. group, with 12 rats in each group. Rats in MPEs group were orally treated with 16 mg/kg/d MPEs, while rats in MPEs+Que. group were orally treated with 16 mg/kg/d MPEs and 50 mg/kg/d Que. Rats in the Que. and control groups were respectively orally administrated with 50 mg/kg/d Que. and the isodose excipient (0.5% sodium carboxymethyl cellulose). The intervention lasted 91 days. All animals were deeply euthanized with isoflurane. About 10 mL blood of each anesthetized rat was collected from the femoral artery, then centrifuged at 4500*g* for 10 min to get serum. Anogenital distance of each rat was measured, and testes were collected and weighed. Relative weight of organs was calculated by the formula: Organ weight (g)/Body weight (g) × 100%. One testis of each rat was fixed in 10% neutral buffered formalin solution for hematoxylin-eosin (H&E) staining and immunohistochemistry. The serum and the other testes were stored at −80 °C for further analyses. All animal work passed the ethical review of experimental animal welfare in Wenzhou Medical University (Approval Number: wydw2019–0050).

2.3. Examination of serum sex hormones

Commercial ELISA kits for rats were used to measure testosterone, LH, FSH, dehydroepiandrosterone, androstenedione, estrone, and dihydrotestosterone levels in serum. Each sample was performed for two parallel reactions. In all cases, the assay kits were operated strictly in accordance with the instructions.

Table 1
Antibody information.

Antibody	Host species	Vendor (CAT No.)	Dilution	
			WB	IHC
PIWIL1	rabbit	ABclonal (A2150)	1:1000	1:200
PIWIL2	mouse	Santa Cruz (sc-377,347)	1:1000	1:200
StAR	mouse	Santa Cruz (sc-166,821)	1:1000	1:200
CYP11A1	rabbit	Beyotime (AF6636) Abcam (ab272494)	1:1000	1:200 1:5000
CYP17A1	rabbit	BOSTER (A00615–3)	1:1000	1:150
17β-HSD	rabbit	Bioworld (BS72641)	1:1000	1:200
CYP19A1	rabbit	Affinity Biosciences (DF3564)	1:1000	1:200
GAPDH	rabbit	Bioworld (AP0063)	1:1000	–

WB, western blot; IHC, Immunohistochemistry.

2.4. Observation of sperm morphology

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 per rat (1200 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. Histopathological observation of testes

The testes tissue was fixed in 10% neutral-buffered 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, and 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 with xylene, and mounting with neutral gum, the sections were finally viewed under a light microscope (DM4000 B LED, Germany).

2.6. Western blot assay

About 50 mg testes tissue was placed in a 1.5-mL Eppendorf (EP) tube, with the addition of precooled radioimmunoprecipitation assay (RIPA) lysate and PMSF protease inhibitor (RIPA: PMSF = 100:1) (Beyotime, Shanghai, China). The tissue samples were homogenized on ice with IKA T10 high speed disperser (Staufen, Germany). After standing on ice for 30 min, the homogenates were centrifuged at 14000*g* for 20 min at 4 °C. Then the supernatants were collected, and the protein concentrations were determined by a BCA method (Beyotime, Shanghai, China). In the aftermath of denaturation, the proteins were fractionated by SDS-PAGE, meanwhile, a pre-stained molecular weight marker (10–180 kD, Beyotime, Shanghai, China) was simultaneously loaded to monitor electrophoresis and identify molecular weights. The fractionated proteins were electroblotted to PVDF membranes, then these membranes were blocked with 5% non-fat skimmed milk at room temperature for 2 h and then incubated with diluted primary antibodies (the diluted concentrations were showed in Table 1) at 4 °C overnight followed by incubation with 1:1000 HRP secondary antibodies for 2 h. ECL reagent was used for detecting the chemiluminescent proteins, and ChemiDoc XRS+ system (Bio-Rad) was used for developing the blots. GAPDH was used as the internal control.

2.7. Immunohistochemistry

Sections (5 μm) of paraffin-embedded tissue were dewaxed and rehydrated, then antigen was retrieved in 10 mM sodium citrate buffer at 95 °C for 15 min. After quenching the sections with 3% hydrogen peroxide for 15 min at room temperature, they were washed with PBS and sealed with 10% bovine serum albumin for 30 min. Primary antibodies (the diluted concentrations were showed in Table 1) were incubated overnight at 4 °C and secondary antibodies for 30 min at 37 °C. After PBS washing, DAB color development, hematoxylin re-staining, running water returning to blue, dehydration and sealing were carried out in sequence, and the results were observed under the optical microscope (DM4000 B LED, Germany).

2.8. Statistical methods

All statistical analyses were conducted using GraphPad Prism 9. Values were expressed as means and their standard deviations (SD). Shapiro-Wilk tests were performed for checking data normality. Parametric test (a Two-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 Two-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. $P < 0.05$ was considered statistically significant. All P values were two tailed.

3. Results

3.1. The Body Weight, Anogenital Distance, and Testes Weight

As shown in Fig. 2, during the experiment, there was no significant difference in rats' average body weight and testis weight among the groups. Compared with control group, rats in MPEs group had a significant reduction in their anogenital distance ($P < 0.05$). Testes weight and the relative testes weight tended to decrease, although there were no significant differences. Whereas, the anogenital distance, testes weight and the relative testes weight of rats in MPEs+Que. group tended to increase when compared with MPEs group, with a significant increase of anogenital distance ($P < 0.05$).

As shown in Table 2, both Que. and MPEs exerted the main effect for the change of anogenital distance. There were no interactions between Que. and MPEs for the changes of body weight, anogenital distance and testes weight.

3.2. Que Reversed MPEs Induced Histological Alterations of Testicular Tissues

Representative testes were shown in Fig. 3. The testes in the MPEs group were obviously shrunk and smaller than that in control group. However, the testes in MPEs+Que. group were obviously larger than that in MPEs group.

Testicular tissue sections were shown in Fig. 3A-3D. As shown in Fig. 3A and B, the testicular morphology of both control and Que. groups showed normal testicular features, such as intact seminiferous tubules and Leydig cells. As shown in Fig. 3C, the testicular tissue of rats in MPEs group was damaged; for examples, the diameters of some seminiferous tubules were reduced (Fig. 3 C1—C4), exfoliation of germ cells were found in the lumen of some seminiferous tubules, and lipid droplets were found in the testicular interstitium (Fig. 3 C1); the number of germ cells was declined (Fig. 3 C2, and C3), and the spermatogenic cells were arranged disorderly (Fig. 3 C4). As shown in Fig. 3D, in MPEs+Que. group, the seminiferous tubules were similar to those in control group and Que. group with normal spermatogenesis and numerous sperms in the tubules.

3.3. Que Reversed MPEs Induced the Increase Rate of Abnormal Sperm

The abnormal sperm rate in MPEs group was significantly higher than that in control group (43.47% *vs* 5.04%, $P < 0.01$) and the abnormalities of the malformed sperm included decapitation, broken tails along with abnormal head appears without hook (Fig. 4C and Fig. 4E). However, the abnormal sperm rate in MPEs+Que. group was significantly lower than that in MPEs group (25.47% *vs* 43.47%, $P < 0.05$).

3.4. Que Reversed MPEs Induced Decrease of Serum Sex Hormones Levels

As shown in Fig. 5, it was significantly decreased of serum

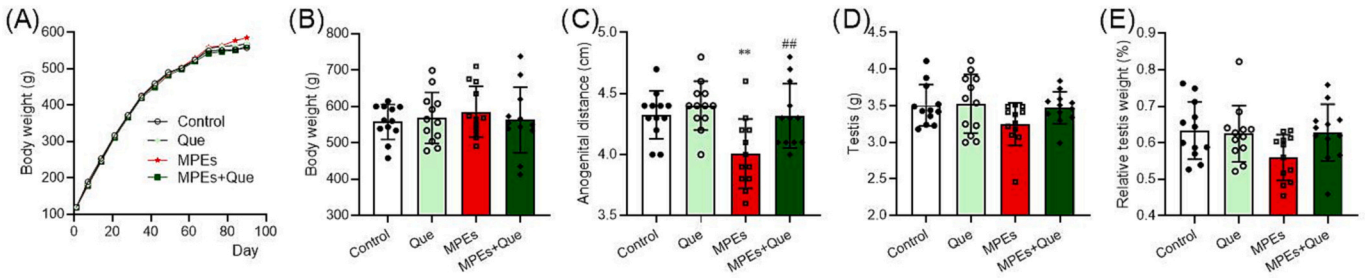

Fig. 2. The body weight, anogenital distance and testes weight. (A) the growth curve of body weight; (B) body weight at necropsy; (C) anogenital distance; (D) testes weight; (E) the relative testes weight; $^{**}P < 0.01$ *vs* control group; $^{\#\#}P < 0.01$ *vs* MPEs group.

Table 2
The main effect, and interaction between Que. and MPEs for the changes of body weight, anogenital distance and testes weight.

	F (*P* value)		
	Que	MPEs	MPEs×Que
Body weight	0.079 (0.780)	0.279 (0.600)	0.653 (0.423)
Anogenital distance	7.733 (0.008)	8.420 (0.006)	2.865 (0.098)
Testes weight	1.909 (0.174)	3.203 (0.080)	1.353 (0.251)
The relative testes weight	2.318 (0.135)	2.249 (0.141)	2.672 (0.109)

testosterone, LH, FSH, dehydroepiandrosterone, androstenedione, dihydrotestosterone and estrone in MPEs group when comparing with control group ($P < 0.05$). However, the serum levels of testosterone, FSH, dehydroepiandrosterone, dihydrotestosterone and estrone in the MPEs+Que. group were significantly higher than those in the MPEs group ($P < 0.05$).

The main effect, and the interaction between Que. and MPEs were showed in Table 3. Que. exerted the main effect for the changes of serum testosterone, FSH, dihydrotestosterone and estrone, while MPEs exerted the main effect for the changes of serum LH, dehydroepiandrosterone, androstenedione and estrone. There were interactions between Que. and MPEs for the changes of testosterone, FSH, dihydrotestosterone and estrone.

3.5. Que Reversed MPEs Induced Changes of Testicular PIWIL1 and PIWIL2

PIWI proteins, such as PIWIL1 and PIWIL2, are germline-specifically expressed in males, and 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 (Aravin et al., 2007; Kojima et al., 2009; Zheng et al., 2010). PIWI proteins could be used as indicators for male reproductive function (Xia et al., 2023). The results of Western blot analysis showed that, compared with control, PIWIL1 was up-regulated in MPEs group ($P < 0.01$), as shown in Fig. 6A-6C, while PIWIL2 was down-regulated in MPEs group ($P < 0.05$). Whereas, compared with MPEs group, PIWIL1 was down-regulated in MPEs+Que. group ($P < 0.01$). Moreover, the results of immunohistochemistry were consistent with the results of Western blot analysis. As shown in Fig. 6D, compared with control, PIWIL1 was up-regulated, while PIWIL2 was down-regulated in MPEs group. Nevertheless, PIWIL1 was down-regulated compared to MPEs group, while PIWIL2 was up-regulated in MPEs+Que. group.

The main effect, and the interaction between Que. and MPEs were showed in Table 4. Que. exerted the main effect for the expression changes of PIWIL1, while MPEs exerted the main effect for the expression changes of PIWIL1 and PIWIL2. There were interactions between Que. and MPEs for the expression changes of PIWIL1.

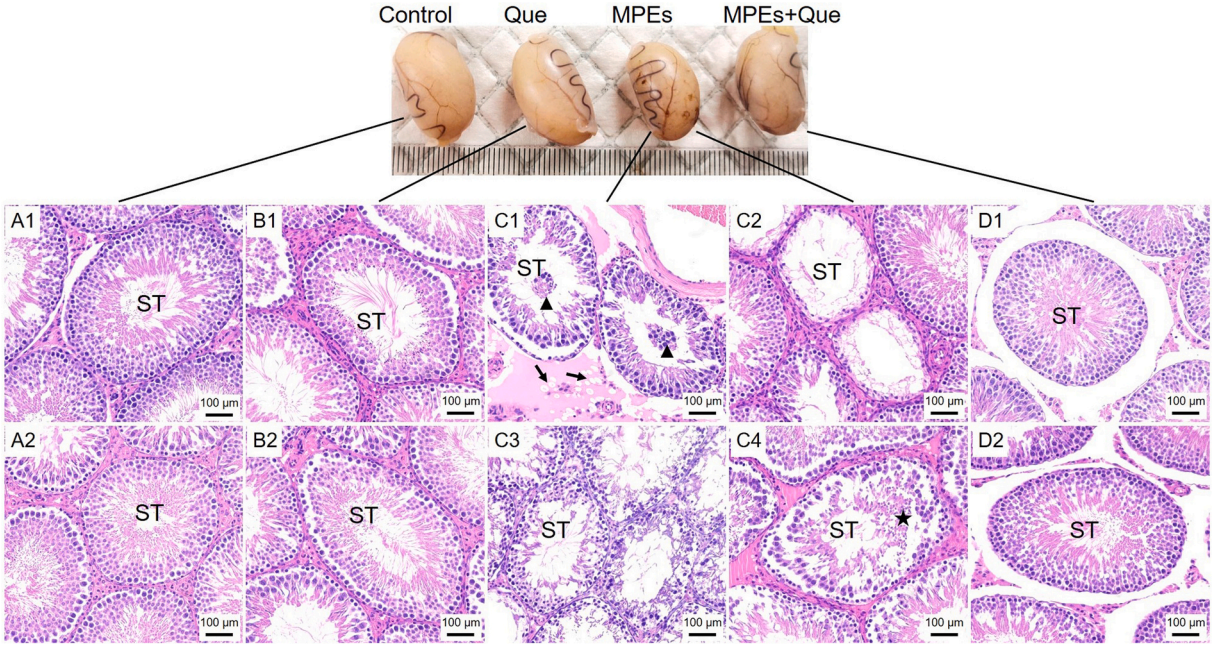

Fig. 3. Testicular morphology and H&E staining in rats. (A) Control group; (B) Que. group; (C) MPEs group; (D) MPEs+Que. group. ST, seminiferous tubule. →, lipid droplet; ▲, spermatids exfoliation; ★, disordered arrangement of spermatogenic cells; ×200, bar = 100 μm.

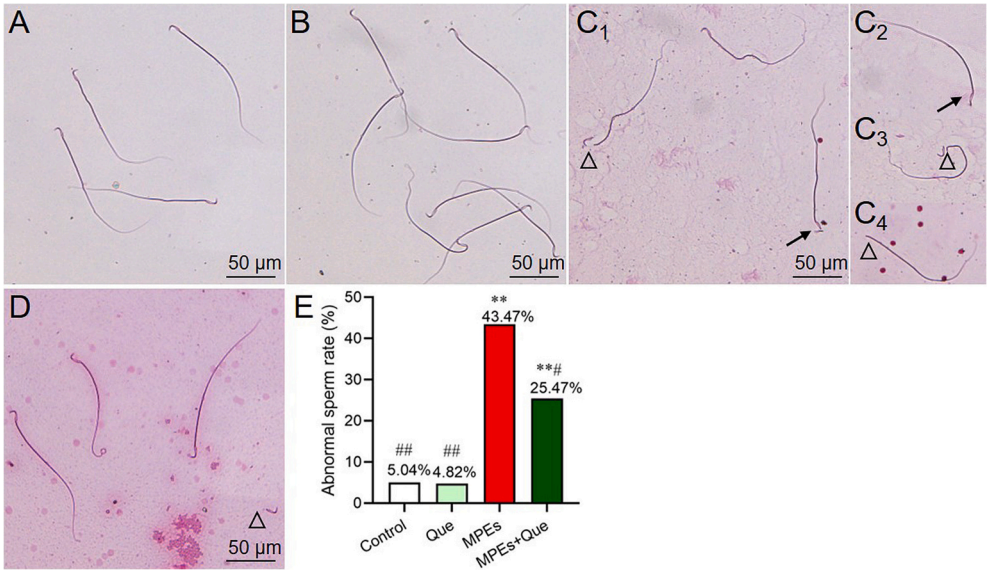

Fig. 4. Sperm morphology of testes in rats. (A) Control group; (B) Que. group; (C) MPEs group; (D) MPEs+Que. group; (E) abnormal sperm rate; Δ , sperm decapitation or broken tails; → , sperm abnormal head appears without hook; $^{**}P < 0.01$ *vs* control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* MPEs group.

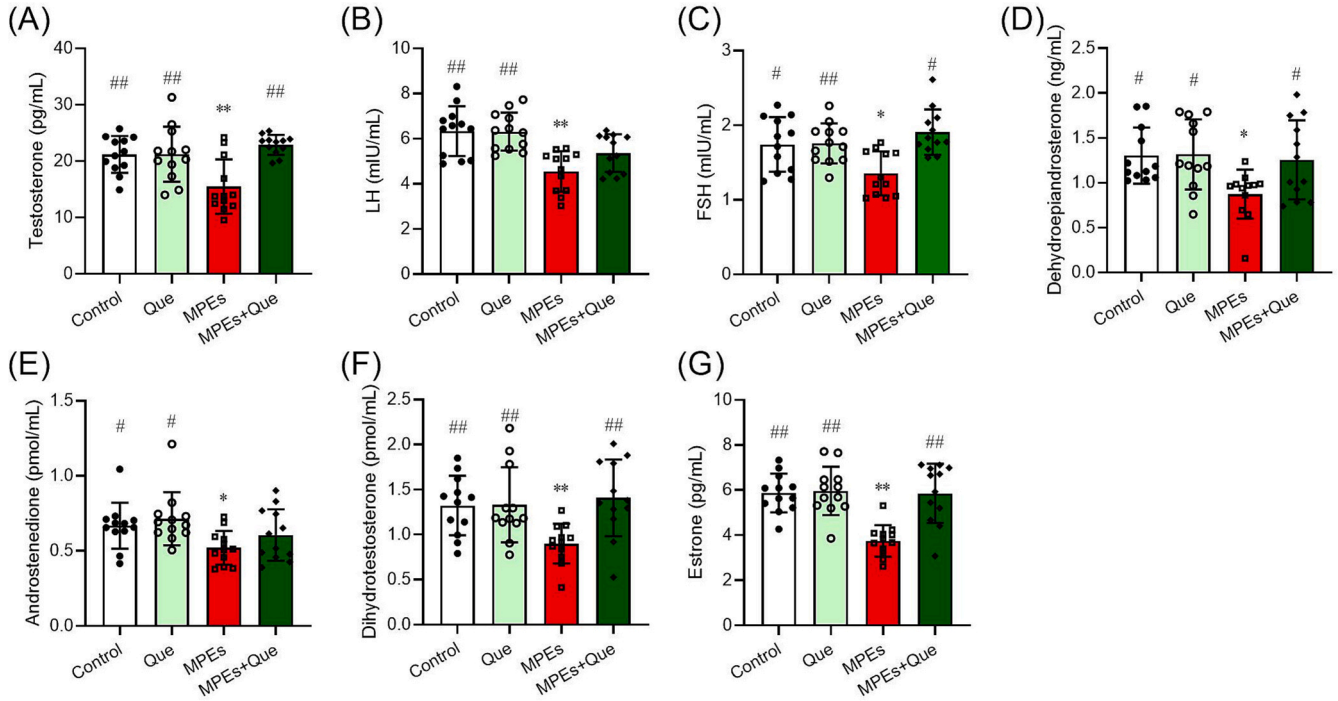

Fig. 5. Serum levels of sex hormones. (A) Testosterone; (B) LH; (C) FSH; (D) Dehydroepiandrosterone; (E) Androstenedione; (F) Dihydrotestosterone; (G) Estrone; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* MPEs group.

3.6. Que Reversed MPEs Induced Alterations of Proteins in Testosterone Biosynthesis

As shown in Fig. 7, the expression levels of StAR, CYP11A1, and CYP17A1 were significantly increased in MPEs group when comparing with control group ($P < 0.05$), while the expression levels of 17β-HSD and CYP19A1 were decreased in MPEs group ($P < 0.05$). Whereas, compared with MPEs group, the expression levels of StAR and CYP11A1 were down-regulated, 17β-HSD and CYP19A1 were up-regulated in MPEs+Que. group ($P < 0.05$).

The main effect, and the interaction between Que. and MPEs were showed in Table 5. Que. exerted the main effect for the expression changes of StAR, CYP11A1, 17β-HSD and CYP19A1, while MPEs exerted the main effect for the expression changes of CYP11A1, CYP17A1, 17β-HSD and CYP19A1. There were interactions between Que. and MPEs for the expression changes of StAR and CYP11A1.

The results of immunohistochemical experiments were in line with the results of western blot. As shown in Fig. 8, most of the seminiferous tubules were at stage VIII and stage XII. Compared with control group, immunohistochemistry revealed that the expression levels of StAR, CYP11A1 and CYP17A1 were elevated, while the expression levels of 17β-HSD and CYP19A1 were reduced in MPEs group. Nevertheless, compared with MPEs group, the expression levels of StAR, CYP11A1 and CYP17A1 were decreased, while the expression levels of 17β-HSD and

Table 3
The main effect, and interaction between Que. and MPEs for the changes of sex hormones.

Sex hormones	*F* (*P* value)		
	Que	MPEs	MPEs×Que
Testosterone	10.99 (0.002)	3.23 (0.079)	10.69 (0.002)
LH	2.184 (0.147)	26.223 (0.000006)	2.247 (0.126)
FSH	10.006 (0.003)	1.761 (0.191)	9.057 (0.004)
Dehydroepiandrosterone	3.650 (0.063)	5.562 (0.023)	3.145 (0.083)
Androstenedione	2.122 (0.152)	8.048 (0.007)	0.190 (0.665)
Dihydrotestosterone	6.260 (0.016)	2.788 (0.102)	5.843 (0.020)
Estrone	14.293 (0.0005)	14.741 (0.0004)	11.997 (0.001)

CYP19A1 were increased in the MPEs+Que. group.

4. Discussion

PEs are ubiquitous in the environment, posing a risk to human health. This study simulating people chronic exposure to the mixture of PEs at low-dose, explored the possible mechanism of MPEs-induced male reproductive toxicity and the protective effect of Que. Earlier studies estimated the total PEs exposure levels of people in China ranging from 34 to 159 mg/kg/day (Gao et al., 2018; Gao et al., 2017). Then, based on the maximum exposure level of total PEs and the safety factor between humans and rats, the exposure dose of MPEs was set at 16 mg/kg/d. Adolescence is a crucial period for the development of the male reproductive system, thus adolescent SD rats were used in this study. According to the previous studies (Khazaeel et al., 2022; Khodabandeh et al., 2021; Ndufeiya-Kumasi et al., 2022), including ours (Xia et al., 2023), Que. dosage was set at 50 mg/kg/d ultimately. After 91 days of continuous intervention, Que. alleviated male reproductive injuries caused by 16 mg/kg/d MPEs in rats.

Previous studies have shown that PEs exposure induced the development of testicular dysgenesis syndrome, such as decrease of testes weight, alterations of testicular morphology, and increase of abnormal sperm morphology (Hosseinzadeh et al., 2022; Liu et al., 2023; Xu et al., 2021). Alterations of organs' weight are intuitive indicators for the organs' injuries. In this study, the decrease of testes weight suggested that MPEs exposure might cause damage to testes in rats. The shortened anogenital distance in MPEs group also indicated that male fertility was impaired in rats (Hsu et al., 2021). Furthermore, H&E morphological analysis revealed atrophy of seminiferous tubule and reduction of spermatogonia and spermatids in MPEs group. The results of sperm smear showed that MPEs exposure significantly increased the abnormal sperm rate. All these results proved that MPEs exposure caused male reproductive damage in rats, which were in accordance with the previous studies (Han et al., 2021; Xia et al., 2023; Ye et al., 2022). As reported, body weight, testes weight, sperm count, and motility were significantly declined following the mice oral exposure to 2 g/kg/d DEHP for 2 weeks (Hosseinzadeh et al., 2022). DBP exposure (500 mg/kg/d, 4 weeks, intraperitoneal injection) resulted in testicular pathological damage, decreased sperm vitality and increased abnormal sperm rate in rats (Başak Türkmen et al., 2022). On the contrary, these

Table 4
The main effect, and interaction between Que. and MPEs for the expression changes of PIWI proteins.

PIWI proteins	*F* (*P* value)		
	Que	MPEs	MPEs×Que
PIWIL1	34.685 (0.0007)	59.606 (0.00001)	33.626 (0.00009)
PIWIL2	0.020 (0.890)	58.987 (0.000006)	4.029 (0.068)

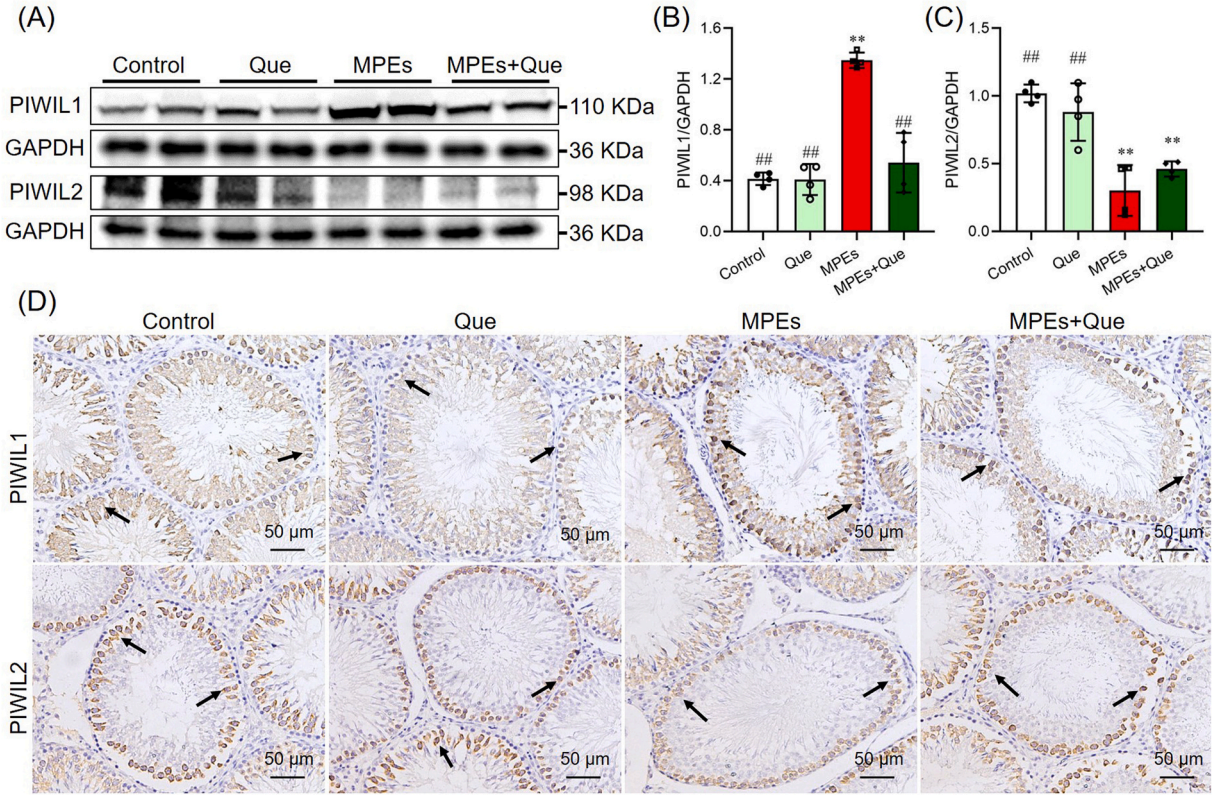

Fig. 6. The expression levels of PIWIL1 and PIWIL2 in rat testes. (A) Representative Western blots images; Protein levels of PIWIL1 (B) and PIWIL2 (C) were quantified and normalized to GAPDH ($n = 4$, mean ± SD). (D) Immunohistochemistry imaging of PIWIL1 and PIWIL2 in rat testes. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* MPEs group.

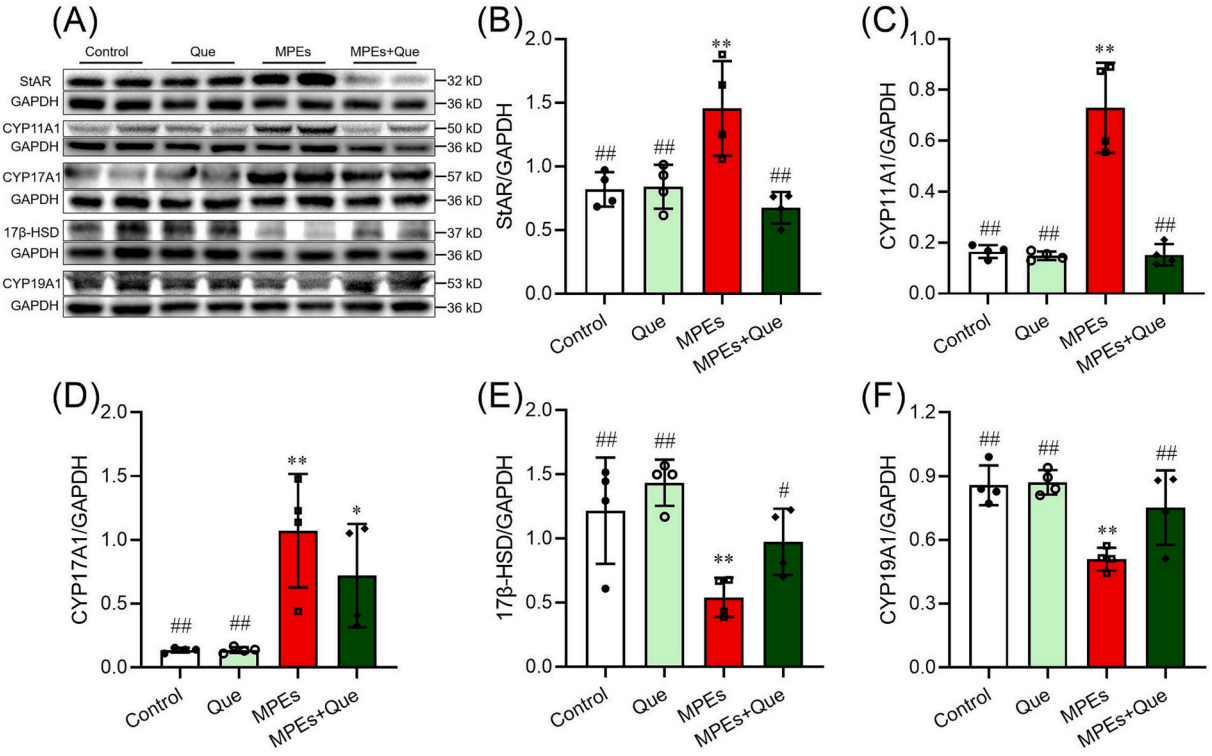

Fig. 7. The expression levels of testicular steroidogenic proteins. (A) Representative Western blot images; Protein levels of StAR (B), CYP11A1 (C), CYP17A1 (D), 17β-HSD (E), and CYP19A1 (F) were quantified and normalized to GAPDH ($n = 4$, mean ± SD). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* MPEs group.

Table 5
The main effect, and interaction between Que. and MPEs for the expression changes of steroidogenic proteins.

Steroidogenic proteins	*F* (*P* value)		
	Que	MPEs	MPEs×Que
StAR	11.461 (0.005)	4.388 (0.058)	12.726 (0.004)
CYP11A1	41.647 (0.00003)	38.208 (0.00005)	37.148 (0.00005)
CYP17A1	1.352 (0.268)	25.358 (0.0003)	1.366 (0.265)
17β-HSD	5.498 (0.037)	17.376 (0.001)	0.574 (0.463)
CYP19A1	5.753 (0.034)	18.996 (0.001)	4.563 (0.054)

parameters were reversed with the presence of Que. in the present study, indicating that Que. ameliorated MPEs-induced male reproductive toxicity.

PIWIL1/2 are expressed in both spermatocytes and spermatids, which interacts with P-element-induced wimpy testis (PIWI)-interacting RNAs (piRNAs) and plays an crucial role in spermatogenesis (Nagirnaja et al., 2021). As aforementioned, PIWIL1/2 could be used as indicators for male reproductive function. PIWIL1 is specifically expressed in germ cells, and loss of PIWIL1 indicated that the germ cells failed to enter the differentiation pathway (Chen et al., 2013). In the study, long-term exposure to low-dose MPEs led to an increase of PIWIL1. This was in accordance with the previous studies, which argued that β-cypermethrin (Zhang et al., 2018) and clotrimazole (Baudiffier et al., 2013) exposure increased testicular PIWIL1 in zebrafish, causing a significant decrease of the testosterone level, and arrest of spermatogenesis. MPEs exposure caused damage to spermatogenesis, which might induce an increase of PIWIL1 with the negative feedback regulation. PIWIL2 is required for germline stem cell self-renewal, and PIWIL2 deficiency leads to defects in meiosis and germ cell survival (Fang et al., 2019). The down-regulation of PIWIL2 in MPEs group was in line with our previous study (Xia et al., 2023), which argued that exposure to MPEs for 30 days at 900 mg/kg/d down-regulated testicular PIWIL2, and induced severely testicular damage in rats. Interestingly, Que. reversed these parameters of reproductive function, added evidence that Que. ameliorated MPEs-induced male reproductive toxicity.

The decrease of testosterone caused by PEs has been presumed to be an upstream mechanistic event in PEs' male reproductive toxicity (Wang et al., 2021b; Zhao et al., 2022). Testosterone is essential for maintaining lifelong spermatogenesis and male fertility, and its production is regulated by the hypothalamus-pituitary-testicular axis (Sun et al., 2022). This axis involves the production of gonadotropin releasing hormone from the hypothalamus that induces the secretion of FSH and LH from the pituitary gland. FSH and LH are gonadotropins that act on the gonads to induce synthesis of sex hormones and promote spermatogenesis (Hou et al., 2020). Under normal physiological conditions, the secretion of various hormones is relatively constant, and increased or decreased secretion of a hormone would affect spermatogenesis and lead to reproductive dysfunction (Kallsten et al., 2022a; Kallsten et al., 2022b). In this study, the decrease of serum LH, FSH and testosterone in MPEs group indicating that MPEs disrupted the function of hypothalamic-pituitary-testis axis. On the other hand, the levels of testosterone, LH and FSH were restored by Que. treatment, suggested that Que. improved the disorder of hypothalamic-pituitary-testis axis induced by MPEs.

As previously mentioned in this text, testosterone is the principal androgen, and maintains normal reproductive function in male mammals. As a matter of fact, >90% of testosterone is synthesized by Leydig cells *via* steroid hormone metabolism (Chen et al., 2021). In this pathway, StAR is responsible for the translocation of cholesterol from the cytosol into mitochondria (Zhao et al., 2021). Then a series of enzymes, such as CYP11A1, CYP17A1 as well as 17β-HSD, catalyze the conversion of cholesterol to testosterone, which is catalyzed into estrogen by CYP19A1 under the normal physiological function (Verma et al., 2020; Zhao et al., 2021).

As mentioned earlier, exposure to PEs could alter steroidogenic proteins in steroid hormone metabolism and lead to a decrease in

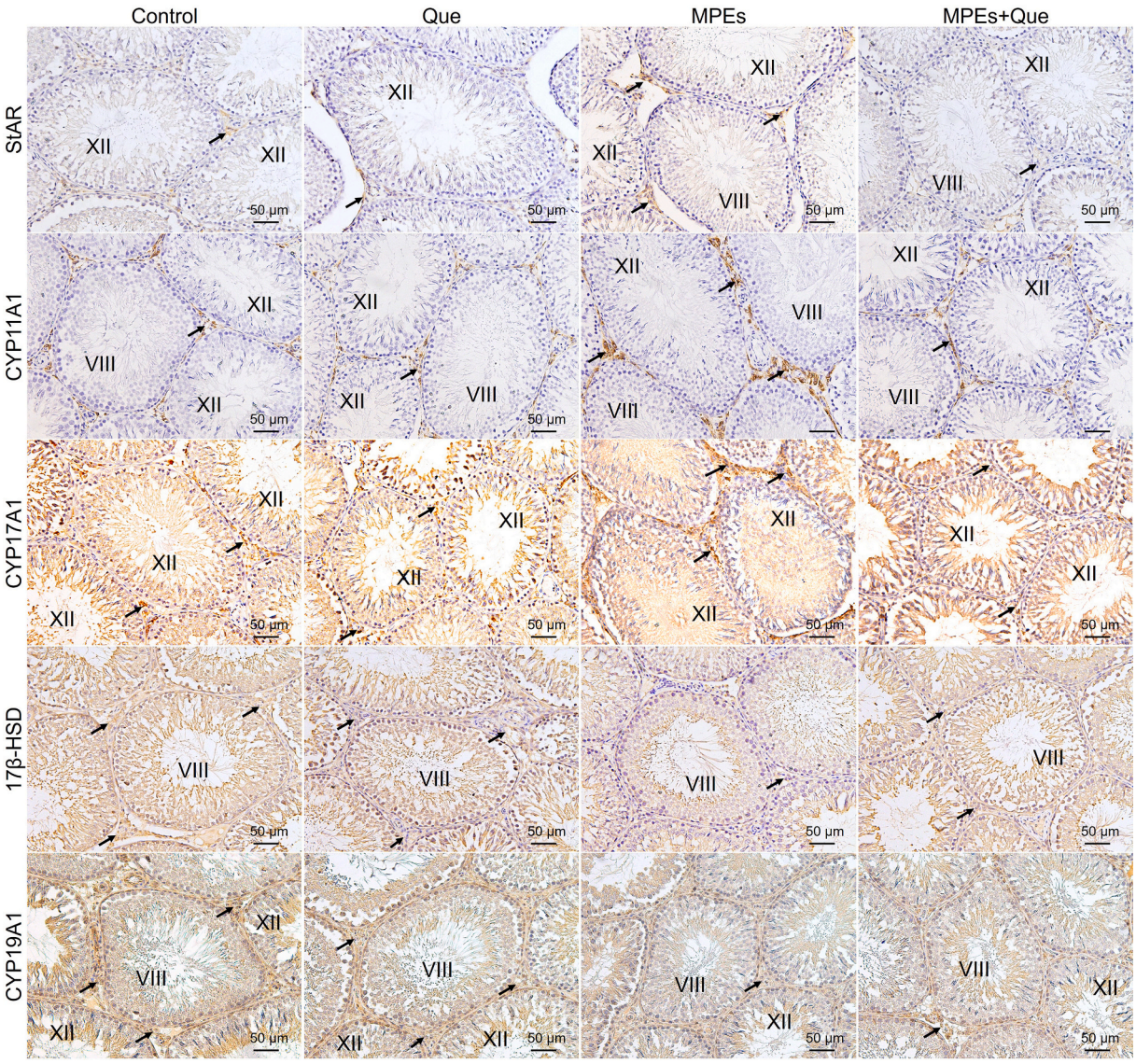

Fig. 8. The expression of testicular steroidogenic proteins in immunohistochemical experiments. There are 14 stages in the spermatogenic cycle in rats (Creasy, 1997). Most of the seminiferous tubules were at stage VIII and stage XII in this study.

testosterone. For example, DBP (100 mg/kg/d, intragastric administration, 5 weeks) decreased serum testosterone, and increased the expression of testicular CYP11A1, HSD3β2 and CYP17A1 in mice (Kallsten et al., 2022a). Oral exposure to the mixture of BBP, DBP and DEHP (900 mg/kg/d, 30 days) also decreased serum testosterone, and increased the expression of StAR, CYP11A1 and CYP17A1 in rat (Xia et al., 2023). In this study, the up-regulation of testicular StAR, CYP11A1 and CYP17A1, while down-regulation of testicular 17β-HSD and CYP19A1 in MEPs group, indicated that MPEs exposure disrupted the metabolism of steroid hormones. Testosterone, dehydroepiandrosterone, androstenedione, dihydrotestosterone and estrone are the intermediate products of steroid hormone metabolism. The decreases of these serum sex hormones in MPEs group further proved that MPEs exposure disturbed metabolism of steroid hormones. The schematic illustration of MPEs exposure induced male reproductive injuries was showed in Fig. 9, that was, MPEs disturbed steroid hormone metabolism, and inhibited testosterone synthesis and secretion, thus caused damage to male reproductive function in rats. On the other hand, Que. reversed the changes of steroidogenic proteins, and the changes of intermediate products of steroid hormone metabolism induced by low-dose MPEs exposure (16 mg/kg/d) in rats, indicating that Que. improved steroid hormone metabolism. Our previous study also showed that Que. improved high-dose (900 mg/kg/d, 30 days) MPEs induced disruption of steroid hormone metabolism in rats (Xia et al., 2023).

This present study explored the effects of MPEs and Que. on male reproductive system, such as the morphology of testes and sperms, and sex hormones, which were the indirect indicators of male fertility. Whereas, the mating assays and sperm function tests could assess male fertility directly in real-world, which should be performed in the further research to investigate the protective effect of Que. against MPEs induced reduction of male fertility. Additionally, the protective mechanism of Que. on male reproductive function requires a deeper investigation, and further verification is better to be performed through *in vitro* experiments.

In summary, chronic exposure to low-dose (16 mg/kg/d) MPEs disturbed steroid hormone metabolism, and caused male reproductive injuries in rats; Que. inhibited MPEs induced male reproductive toxicity by improving the regulation of steroid hormone metabolism, and promoting the synthesis and secretion of testosterone. The results of this study indicate that Que. can be used as a nutritional supplement to prevent PEs exposure induced male reproductive injuries. Que-enriched foods, such as tea, vegetables (onions, capers, shallots) and fruits

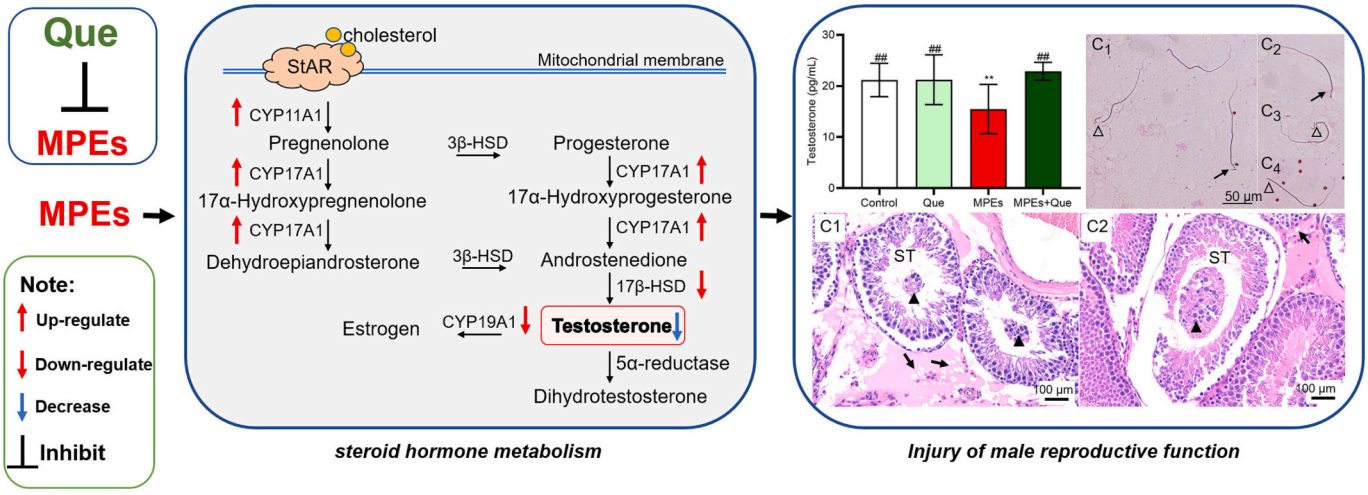

Fig. 9. The schematic illustration. Que. inhibits MPEs induced disruption of steroid hormone metabolism in male rats. Long-term low-dose MPEs exposure changed the expression of key proteins in steroid hormone metabolism, disturbed steroid hormone metabolism, and inhibited testosterone synthesis and secretion, thus caused damage to male reproductive function in rats. Que. alleviated MPEs induced decrease of testosterone by improving testosterone biosynthesis, and inhibited the male reproductive toxicity of MPEs.

(berries, grapes, apples), are advisable and may reduce reproductive hazards of PEs exposure.

Authors contributions

Li-Lan Liu: Investigation, Methodology, Validation, Visualization, Writing-Original Draft; Jun-Zhe Yue: Investigation, Validation, Visualization; Zhen-Yu Lu: Investigation; Ru-Ya Deng: Investigation; Can-Can Li: Investigation; Ye-Na Yu: Investigation; Wen-Jin Zhou: Investigation; Min Lin: Data Curation, Supervision; Jiaming Liu: Supervision, Writing-Review & Editing; Hai-Tao Gao: Conceptualization, Methodology, Writing-Review & Editing, Resources, Funding acquisition; Ling-Zi Xia: Data Curation, Supervision, Writing-Review & Editing; Funding acquisition.

CRediT authorship contribution statement

Li-Lan Liu: Writing – original draft, Visualization, Validation, Methodology, Investigation. **Jun-Zhe Yue:** Visualization, Validation, Investigation. **Zhen-Yu Lu:** Investigation. **Ru-Ya Deng:** Investigation. **Can-Can Li:** Investigation. **Ye-Na Yu:** Investigation. **Wen-Jin Zhou:** Investigation. **Min Lin:** Supervision, Data curation. **Hai-Tao Gao:** Writing – review & editing, Resources, Methodology, Funding acquisition, Conceptualization. **Jiaming Liu:** Writing – review & editing, Supervision. **Ling-Zi Xia:** Writing – review & editing, Supervision, Funding acquisition, Data curation.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Data availability

Data will be made available on request.

Acknowledgements

This study was supported by Zhejiang Provincial Natural Science Foundation of China (Grant No. LY22H260002), National Natural Science Foundation of China (Grant No. 81903321), and Wenzhou Science & Technology Bureau Project (Grant No. Y2023022).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2024.116816.

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