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FULL LENGTH ARTICLE

DEHP exposure destroys blood-testis barrier (BTB) integrity of immature testes through excessive ROS-mediated autophagy

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Abstract Di-(2-ethylhexyl) phthalate (DEHP), is known to impair testicular functions and reproduction. However, its effects on immature testis Blood-testis barrier (BTB) and the underlying mechanisms remain obscure. We constructed a rat model to investigate the roles of autophagy in BTB toxicity induced by DEHP. Sprague–Dawley rats were developmentally exposed to 0, 250 and 500 mg/kg DEHP via intragastric administration from postnatal day (PND) 1 to PND 35. Testicular morphology, expressions of BTB junction proteins and autophagy related proteins were detected. In addition, expressions of oxidative stress markers were also analyzed. Our results demonstrated that developmental DEHP exposure induced decreasing organ coefficients of immature testes and severe testicular damage in histomorphology. The expressions of junctional proteins were down-regulated significantly after DEHP treatment. Intriguingly, DEHP simultaneously increased the number of autophagosomes and the levels of autophagy marker LC3-II and p62, suggesting that the accumulated autophagosomes resulted from impaired autophagy degradation. Moreover, the expressions of HO-1 and SOD levels remarkably decreased after DEHP exposure. Vitamins E and C could alleviate the DEHP-induced oxidative stress, reverse the autophagy defect and restore the BTB impairment. Taken together, DEHP

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exposure destroys immature testis blood-testis barrier (BTB) integrity through excessive ROS-mediated autophagy.

Introduction

Di-(2-ethylhexyl) phthalate (DEHP) is a commonly used plasticizer with a diverse range of industrial applications: toys, clothing, food packaging materials and building materials. It is also employed in non-polymer materials such as lacquers, fillers, printing inks, and cosmetics.[1–3] About 1–4 million tons per year were produced and spread worldwide, which makes it one of the most widespread environmental contaminants.[4]

People can be exposed to DEHP through multiple routes: water, indoor air and medical devices.[5] DEHP-exposure has been demonstrated to change kidney and liver function. Additionally, it has also been found to damage the reproductive system and cause male infertility.[6,7] However, its effect on immature testis and the underlying mechanisms of immature testicular toxicity remains unclear.

Stability of immature testicular microenvironment is the premise of spermatogenesis and indispensable for maintaining male reproductive function. The blood-testis barrier (BTB) constituted by tight junctions, adherens junctions and gap junctions between adjoining Sertoli cells plays a pivotal role in spermatogenesis microenvironment. They extend from the base to the apex of seminiferous epithelium, interact directly with developing germ cells throughout spermatogenesis. When junctions proteins expressed abnormally in Sertoli cells, spermatogenic cells cannot manifest differentiation or meiosis, which finally led to impaired sperm production and sterility. Sertoli cells, in particular Sertoli cell–cell and Sertoli-germ cell junctions have recently been identified the targets of environmental toxicants in the testis. Therefore, we speculated that DEHP caused immature testicular toxicity through BTB integrity impairment.[8]

Reactive oxygen species (ROS) has acted as a fundamental mechanism of DEHP-related impairment. Free radicals and ROS like superoxide (O.2), hydroxyl radical (.OH) and hydrogen peroxide (H2O2) are thought to contribute to lipid peroxidation (LPO), DNA damage and protein degradation during oxidative stress in rat model. Further, testes are more prone to oxidative stress as it is a sensitive organ requiring more oxygen. Hence, oxidative stress probably plays an important role in DEHP-induced BTB damage of males.[9,10]

Recently, it has been demonstrated that mitochondrial ROS generation could trigger autophagy through several distinct mechanisms involving Atg4 and the mitochondrial electron transport chain (mETC).[11–13] Several lines of evidence support a role for apoptosis in the toxicity of DEHP, while many questions remain unsolved regarding to autophagy.

As an evolutionary conserved lysosomal catabolic mechanism, autophagy involves in removing damaged and dysfunctional proteins or organelles in response to certain stimuli such as hypoxia and starvation.[14] The process of autophagic degradation is initiated when a portion of the cytosolic component is sequestered in isolation membranes. The isolation membranes elongate and eventually seal to become double-membrane vesicles called autophagosomes that then fuse with lysosomes resulting in degradation of the enclosed components. The process is essential for cell growth, survival, differentiation and the failure of autophagy may lead to abnormal skeletal development neurodegeneration, cardiomyopathies and cancers. Additionally, autophagy is essential for spermatogenesis, especially in the clearance of apoptotic germ cells, Sertoli cells or Leydig cells.[15,16]

A family of genes whose products are involved in the process of autophagy are called autophagy-related genes (ATGs). Nucleation and assembly of the initial membrane are dependent on the complex formed by Beclin1. LC3 has been widely used to monitor the number of autophagosomes as well as autophagic activity. Hence, Beclin1 and LC3 II are commonly used as markers of ongoing autophagy. SQSTM1/p62 is a cargo-transporting protein facilitating autophagosome degradation.

To our knowledge, until now, whether autophagic process participates in the regulation of DEHP-induced testicular toxicity and BTB impairment is still largely unknown and the function of Beclin-1, LC3 and p62 made in the toxicity of DEHP is also unclear. In this study, we mainly focused on the effect of DEHP on the induction of autophagy, ROS and the potential underlying mechanisms.

Materials and methods

Ethics statement

All experiments involving animals were approved by the Ethics Committee of Chongqing Medical University (SCXKYU20110016).

Animals and treatments

All rats used in this study had free access to food and water, and kept under the specific pathogen-free condition (12 h light/12 h dark cycle with humidity of 55 ± 5% at 25 ± 2 °C). Pregnant female Sprague–Dawley (SD) rats (220–300 g) were purchased from Experimental Animal Center of Chongqing Medical University (Chongqing, China). After delivery, 60 postpartum male rats were randomly divided into four groups as follows: Corn oil control group, DEHP 250 mg/kg group, DEHP 500 mg/kg group, DEHP 500 mg/kg + 200 mg/kg.b.w vitamin E + 100 mg/kg.b.w vitamin C

group. Rats were given DEHP (Yuanye Bio-technology Co, Ltd, Shanghai, China) from PND1 to 35 through intragastric daily, DEHP was dissolved in corn oil, Vitamin E were dissolved in corn oil, and vitamin C was dissolved in distilled water. After 35 days of exposure, all rats were sacrificed and testes were immediately isolated.

HE staining

Testicular tissues were fixed in 4% paraformaldehyde and paraffin-embedded. Subsequently, testicular tissues were cut into 4 μm sections, deparaffinized and rehydrated. Slides were dipped into hematoxylin and agitated for 2 min, then rinsed in H2O for 1 min. The slides were subsequently stained with 1% eosin solution for 3 s with agitation. The sections were dehydrated with two changes of 95% alcohol and two changes of 100% alcohol for 3 min each. Finally, the alcohol was extracted with two changes of xylene. Images were acquired on an Olympus microscope (BX40).

Transmission electron microscopy

These experiments were conducted in the Pathology Electron Microscopy Facility of the Chongqing Medical University using standard techniques. Three rats were randomly selected from each group for ultrastructure evaluation.

Immunofluorescence

10 μm Frozen sections of testis obtained with cryostat microtome and Sertoli cells were fixed in 4% PFA for 15 min and permeabilized in 0.1% Triton X-100 in PBS for 10 min. Then sections and cells blocked in 0.5% BSA in PBS for 1 h at room temperature, followed by an overnight incubation of primary antibodies at 4 °C. The primary antibodies used were LC3 (1:100, ab128025, Abcam), p62 (1:200, ab91526, Abcam), SOD (1:200 ab16831; abcam), Anti-Heme Oxygenase 1 (1:200; ab68477; abcam), β-catenin (1:100, ab32572, Abcam), Cx43 (1:100, ab11370, Abcam), and ZO-1 (1:100, 61−7300, Invitrogen). Following a PBS wash, the sections were incubated with goat anti-rabbit (CW0159S) or mouse (CW0145S), Cy3 Conjugated (CwBio, China) for 1 h at room temperature. The nuclei were stained with hoechst 33342 (C1022, Beyotime, China) for 1 h. Images were obtained with an A1R Confocal microscopy system (Nikon, Tokyo, Japan) and prepared with Nikon NIS-element AR 4.0 software.

Protein extraction and western blot

Proteins were extracted from rat testes using radioimmunoprecipitation assay reagent (RIPA, P0013B, Beyotime Biotech, China) containing 1% phenylmethanesulfonyl fluoride (PMSF, ST506, Beyotime Biotech, China) to prevent protein degradation. Protein concentrations were measured by BCA method (BCA, P0012, Beyotime Biotech, China). Protein samples were mixed with 5 × sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer and boiled for 5 min before being loaded onto a 10% SDS-PAGE gel. After electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, USA). Membranes were cut according to the marker and incubated in 5% non-fat milk-PBST for 1 h on the shaker at RT to block nonspecific protein binding. The primary antibodies used were SOD (1:1000 ab16831; abcam), Anti-Heme Oxygenase 1 (1:1000; ab68477; abcam), ZO-1 (1:1000; 61−7300, Invitrogen), β-catenin (1:1000; ab32572, Abcam), Cx43 polyclonal rabbit antibody (1:3000, ab11370, Abcam, 1:3000), Beclin1 (1:1000; ab62557; Abcam), LC3 (1:500; ab128025; Abcam), p62 (1:1000; ab91526; Abcam) and β-actin mouse antibody (1:500; TA-09, ZSGB-BIO, China) was used as a control. Membranes were incubated with primary antibodies overnight at 4 °C and then washed in PBST for 3 × 10 min before being incubated with the corresponding secondary antibody. The bands were detected by chemiluminescent reaction (Millipore, USA). Image collection and densitometry analysis were performed by Quantity One version 4.6.2 analysis software.

Statistical analysis

Each experiment was repeated at least three times, SPSS 13.0 (Chicago, USA) was used to conduct statistical analyses. Comparisons between groups were analyzed using an analysis of variance (one-way ANOVA) and differences were considered significant when $p < 0.05$. The data were expressed as the means ± S.E.M.

Results

Organ coefficient of rats after treatment

Table 1 showed a significantly decrease in organ coefficients of testis, epididymis as well as seminal vesicle in the DEHP-treated group ($P < 0.05$) (Table 1, Fig. 1. A, B, C and D).

Histomorphological changes in testis after DEHP exposure

Subsequently, we examined the testis histological morphology after DEHP exposure through HE staining. As shown in Fig. 2, compared with control group, tubule

Table 1 Organ coefficient information after DEHP exposure in different groups. Values are expressed as mean ± SD, n = 6.

Organ coefficient	Group		
	Corn oil control	DEHP 250 mg/kg	DEHP 500 mg/kg
Testis	0.00551 ± 0.00095	0.00358 ± 0.00075	0.00258 ± 0.00052
Epididymis	0.000849 ± 0.000091	0.000665 ± 0.0000099	0.000536 ± 0.000091
Seminal vesicle	0.000538 ± 0.000069	0.000299 ± 0.0000080	0.000225 ± 0.000074

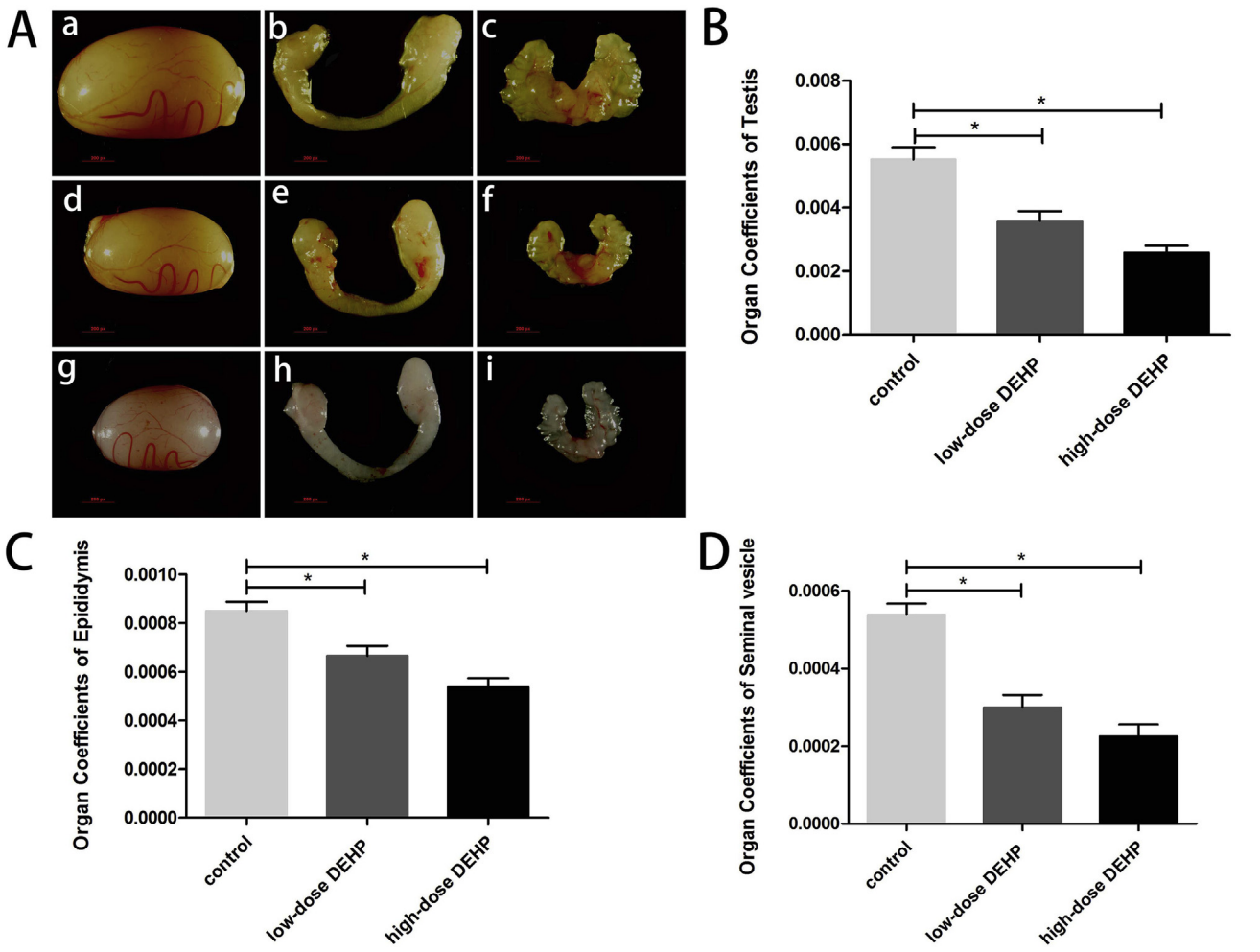

Fig. 1 The Organ Coefficients between group control and group exposure to DEHP. The organ coefficients of testis (A. a, d and g), epididymis (A. b, e and h) and seminal vesicle (A. c, f and i) were significantly decreased in low-dose DEHP group and high-dose DEHP when compared with the control group ($p < 0.05$).

diameters and spermatogenic cells were greatly decreased. Low-dose group showed contraction deformation of seminiferous tubules, hyperchromatic nuclei in lumen and detachment and sloughing of spermatogenic cells into the lumen. This marked histopathological changes with cellular damage was more obvious in high-dose group. The results demonstrated that DEHP exposure can impair spermatogenesis.

BTB integrity was destroyed after DEHP treatment

As we known, blood-testis barrier plays a crucial role in spermatogenesis. Therefore, we determined the expressions of ZO-1, β-catenin and connexin43. We found that β-catenin (Fig. 3 B and C), Cx43 (Fig. 3 E and F) and ZO-1 (Fig. 3 H and I) expressions were conspicuously down-regulated both in DEHP group when compared with control group ($P < 0.05$). β-catenin abundantly located primarily in Sertoli cell layers (Fig. 3. A). Cx43 mostly located in basal compartment of seminiferous epithelium and spermatid layers (Fig. 3. D). ZO-1 located between germ cells of testicular seminiferous epithelium, especially basal compartment (Fig. 3. G). It is shown that Sertoli cell layers of testicular seminiferous epithelium or/and spermatid layers were strongly stained in control group compared to DEHP exposure group (Fig. 3. A, D and G). Furthermore, ZO-1 were observed mis-localization from membrane to cytoplasm after DEHP exposure.

DEHP caused oxidative stress injury of testis

It is demonstrated that DEHP get into the body and produces excessive reactive oxygen species (ROS) to enable increased oxidative stress in cells, which leads to cell death.[17–19] Thus, we assessed oxidative stress levels in rat testis by analyzing testicular SOD, HO-1 expressions by Western blot and Immunofluorescence. We found that SOD (Fig. 4. A) and HO-1 (Fig. 4. D) mainly located in cytoplasm of sertoli cells and spermatogenic cells, and expression levels were decreased significantly in DEHP exposure groups than that in control group (Fig. 4).

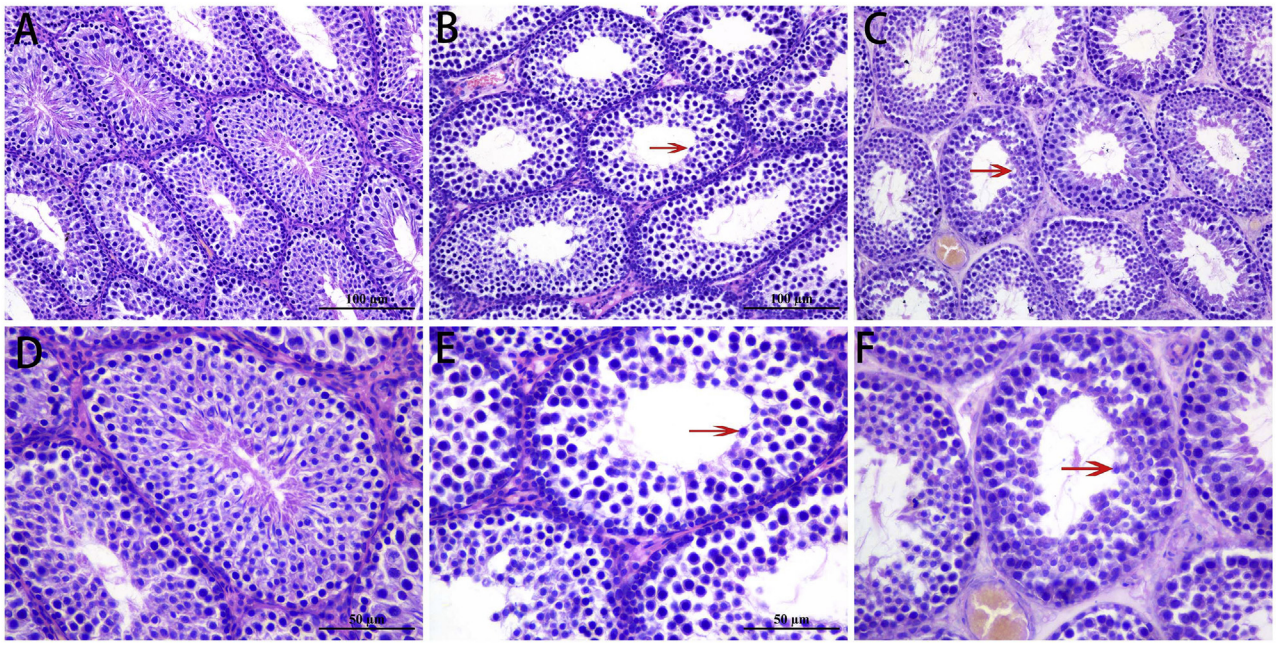

Fig. 2 Testicular histomorphological change of SD rats after treatment with DEHP on PND35. The morphology of spermatogenic cells and Sertoli cells were normal, and elongated spermatids can be seen in seminiferous tubule in corn oil treated group (A and D). Compared with control group, abnormalities were in 250 mg/kg DEHP exposure group (B and E) and 500 mg/kg DEHP exposure group (C and F) with cells types not identifiable, hyperchromatic nuclei, greatly reduced tubule diameters and spermatogenic cells dramatically decreased.

DEHP induced ultrastructural abnormalities in rat testes

Recently, it was also widely accepted that autophagy could be trigger by ROS through several distinct mechanisms.[11,12] Then, we performed transmission electron microscopy of testis in control group and low-dose DEHP. After DEHP exposure, vacuolation, mitochondrial swelling and degeneration, destroyed BTB and autophagosomes accumulation were observed in testis (Fig. 5 d, e, f, g, h and i). Moreover, a number of autophagosomes-like structures (some containing mitochondria and other cytoplasmic contents) were dramatically presented throughout the cytoplasm of sertoli cells and germ cells (Fig. 5 d, e, f, g and h).

DEHP exposure induces the protein expressions of LC3 and p62 in rat testes

Because autophagosomes-like structures is associated with spermatogenesis, we examined the expression levels of Beclin-1, LC3 and p62 in rat testis. We found that LC3 levels (Fig. 6 D and E) and p62 levels (Fig. 6 D and F) were conspicuously upregulated both in DEHP-treated groups compared with control group ($p < 0.05$). Immunofluorescence staining showed that LC3 was amply expressed in Sertoli cell layers and spermatogenic cell layer after DEHP exposure (Fig. 6. A), p62 was located in seminiferous epithelium and spermatid layers (Fig. 6. B), suggesting impaired autophagic degradation. Conversely, Beclin-1, a critical regulator of autophagosome formation were not obviously influenced following DEHP treatment (Fig. 6 D and F). The results demonstrated that DEHP exposure brought autophagy defect and this effect is caused by impaired degradation rather than formation.

Vitamins E and C alleviated the DEHP-induced testis toxiocity

To explore the relationship between oxidative stress and autophagy and whether vitamin C and E could reverse BTB impairment caused by DEHP, we investigated the associated markers expressions. After treatment with vitamin E and C, we found that the expression levels of oxidative stress markers (SOD and HO-1), autophagy-related genes (LC3II/I and p62) and BTB-associated proteins (ZO-1, β-catenin and Cx43) became normally (Fig. 7). Meanwhile, the location of BTB-associated proteins became similar with that in control group (Fig. 7. J and K). Therefore, vitamins E + C supplement normalized the oxidative stress state in testes after DEHP exposure and the restored oxidative stress state rescued the autophagy defect and BTB integrity.

Discussion

Human beings are exposed to DEHP daily from pre-pregnancy to post-puberty, which covers the critical maternal, perinatal, and pubertal periods. Numerous studies demonstrated that, DEHP is rapidly metabolized into mono-2-ethylhexyl phthalate and induces reproductive toxicants, developmental toxicants, reduction in fertility, low sperm density and motility in developing animals.[20] However, the underlying mechanism is not clear. Thus, understanding how DEHP results in sperm damage is a central issue in toxicology.

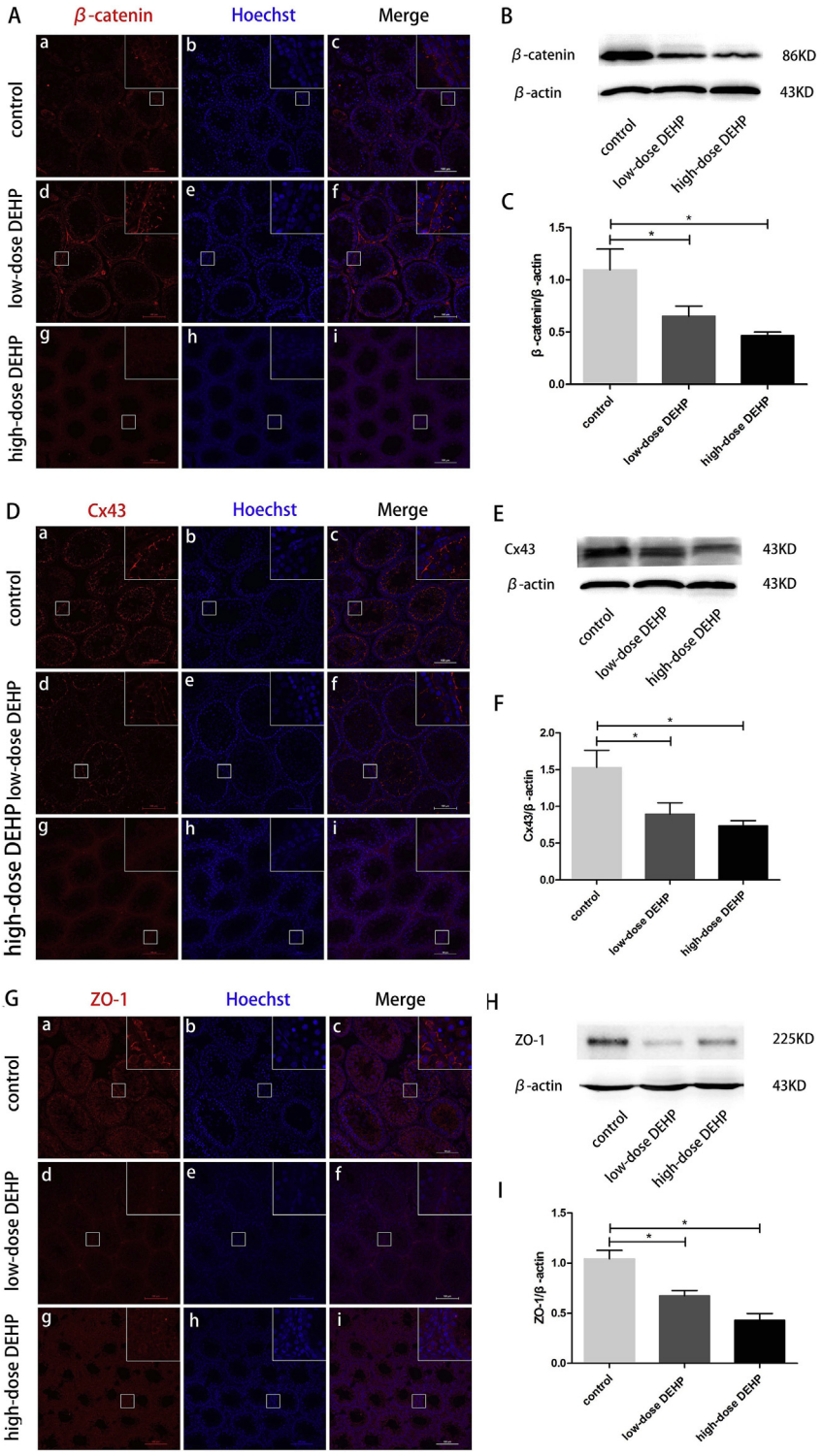

Fig. 3 Alteration of β-catenin, Cx43 and ZO-1 in rat testis after DEHP treatment. Immunofluorescence of β-catenin, Cx43 and ZO-1 in testicular tissues from representative rats was performed. An enhanced β-catenin staining was observed in control group and located between germ cells of testicular seminiferous epithelium, especially basal compartment (A). Cx43 and ZO-1 was richly expressed in control group and located chiefly in spermatid layers and basal compartment of testicular seminiferous epithelium (D and G). β-catenin, Cx43 and ZO-1 expression lowered saliently in both low-dose DEHP-treated group and high-dose DEHP-treated group (A, D and G) compared with control group. In support of this, western blotting analysis demonstrated that β-catenin levels was downregulated in testicular tissues from representative rats treated with different concentration DEHP compared with control group (B, E and H) ($p < 0.05$). Densitometry values of β-catenin was normalized to β-actin. Three sections were analyzed for each sample. * Significantly different, $p < 0.05$. # No significantly different, $p > 0.05$.

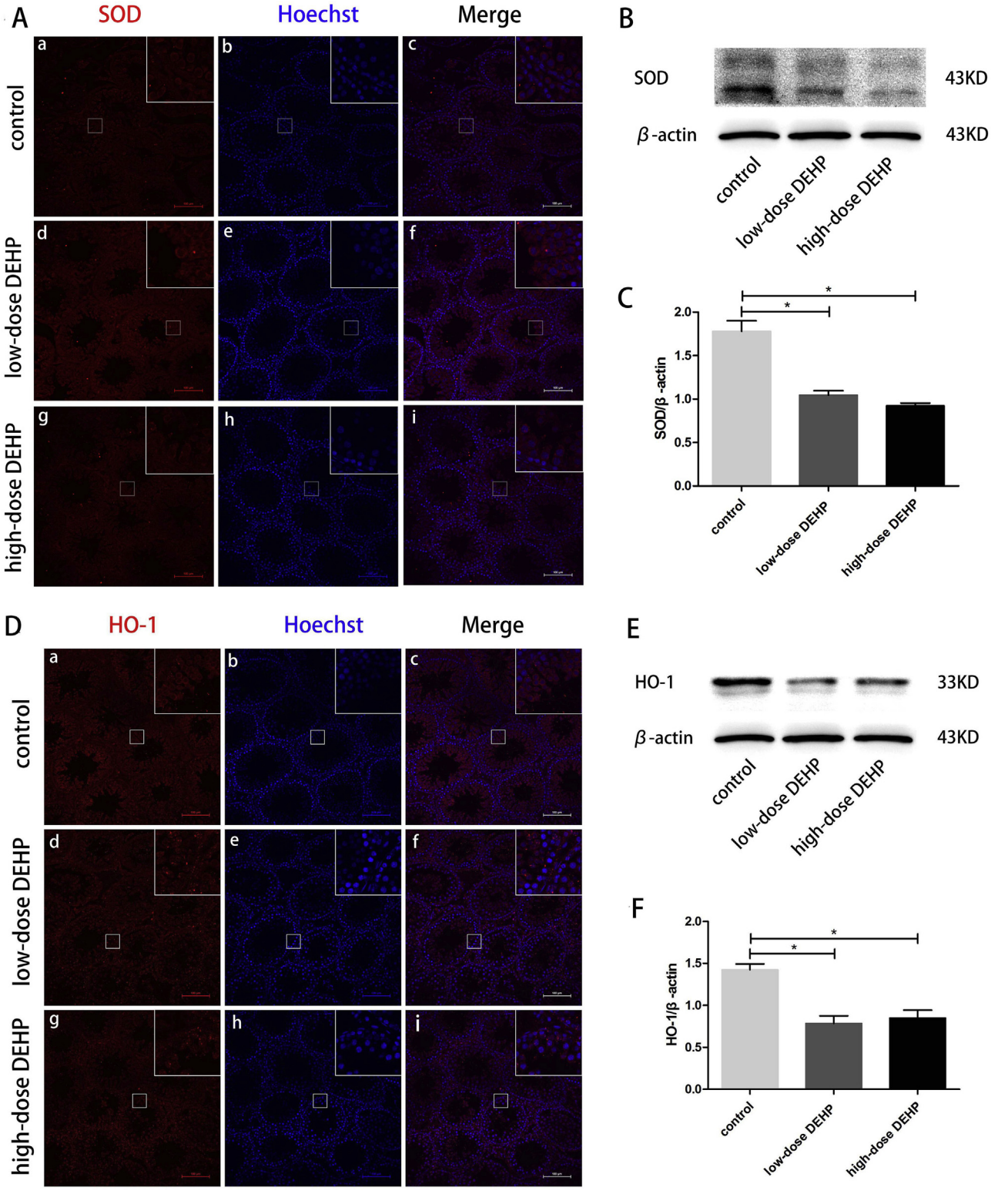

Fig. 4 SOD and HO-1 protein expression in testes of rats after DEHP treatment. Western blotting analysis of SOD and HO-1 in rat testis tissues of control group and DEHP exposure group (250 mg/kg.b.w. DEHP and 500 mg/kg.b.w. DEHP) after treatment were presented. Western blotting analysis demonstrated that SOD and HO-1 expression strongly decreased in DEHP treatment group compared with control group (B and E) ($p < 0.05$). Three sections were analyzed for each sample. * Significantly different, $p < 0.05$. # No significantly different, $p > 0.05$. Immunofluorescence of SOD and HO-1 in testicular tissues from representative rats were also performed. It is shown that the expression of SOD and HO-1 in cytoplasm of sertoli cells and spermatogenic cells were reduced after DEHP exposure (A and D).

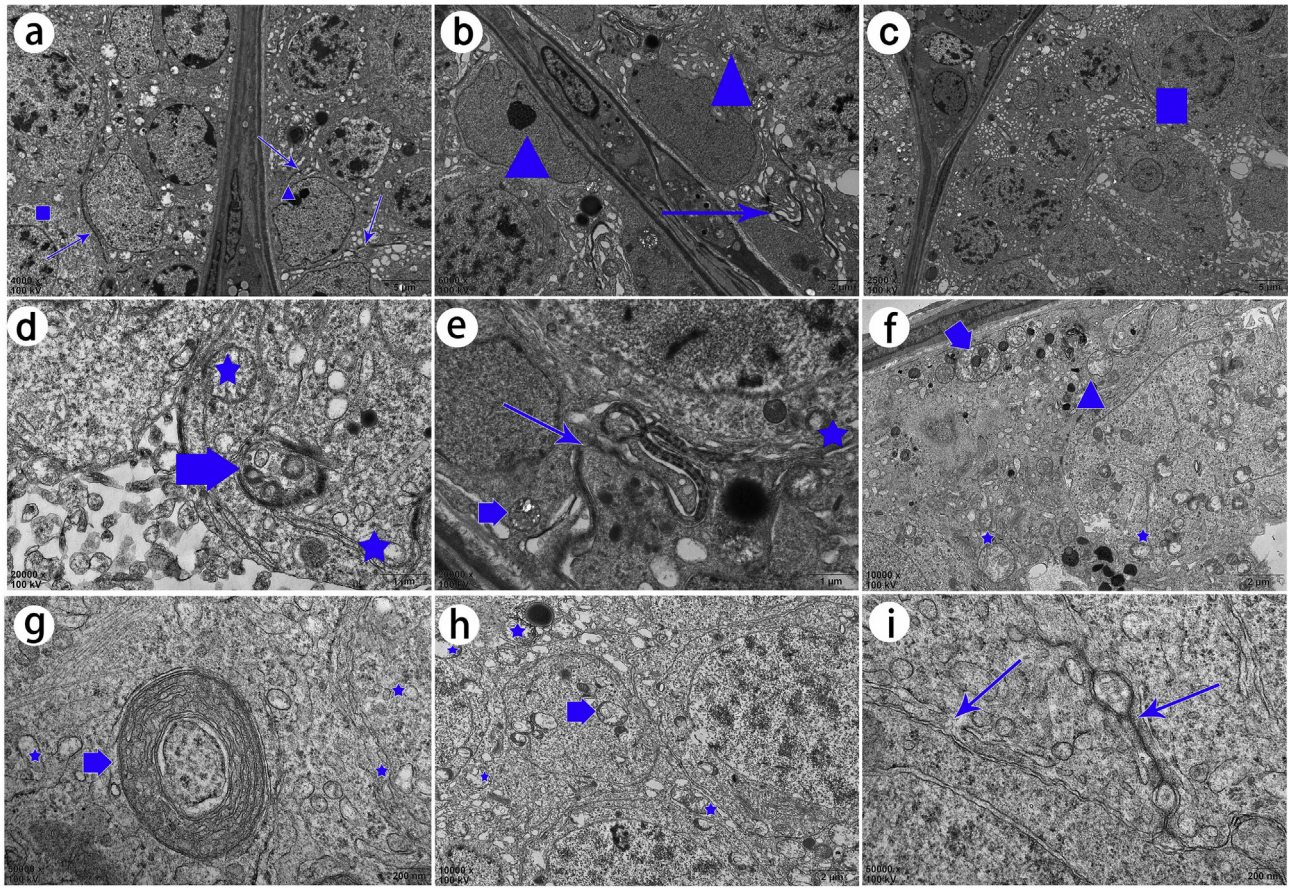

Fig. 5 Testicular ultrastructural abnormalities after treatment with low-dose DEHP. The morphology of spermatogenic cells and BTB which is formed between adjacent sertoli cells (a, b and c) were normal in seminiferous tubule of control group. Impairment of testicular tissue, such as vacuolation, mitochondrial swelling and degeneration, destroyed BTB and autophagosomes accumulation were observed in testicular tissue of DEHP-treated rats (d, e, f, g, h and i). Numerous autophagosomes-like structures were presented throughout the cytoplasm of sertoli cells (d and e) and spermatogenic cell (f, g and h) (a: 4000 ×, b: 6000 ×, c: 2500 ×, d 20000 ×, e: 25000 ×, f: 10000 ×, g: 10000 ×, h: 30000 ×, i: 50000 ×). The thin arrows indicate BTB. The thick arrows indicate autophagosomes and the asterisks indicate swollen mitochondria with the degeneration or loss of cristae. The square indicate the spermatogenic cells and the triangle indicate the sertoli cells.

In the present study, we firstly measured the size of male reproductive organs. Notably, the organ coefficients of testis and epididymis showed significantly reduction as well as seminal vesicle, when compared with the control group. These findings suggested that DEHP caused testicular toxicity in rats.

Then, we examined the change of testicular histomorphology through HE staining, and found that contraction deformation of seminiferous tubules, hyperchromatic nuclei in lumen and detachment and sloughing of spermatogenic cells into the lumen were present in the DEHP-treated group. These results can undoubtedly impair spermatogenesis.

The reason of spermatogenesis impairment in immature testis after DEHP exposure was unclear. BTB is a well-known premise of spermatogenesis, when junctional proteins expressed abnormally in Sertoli cells, spermatogenic cells cannot manifest differentiation or meiosis, which finally led to sterility.[21–23] In this study, BTB-associated proteins such as β-catenin, ZO-1 and Cx43 expressions and locations were abnormal after DEHP exposure. However, when giving vitamin E and C treatment, their expression can became similar with control group.

Various antioxidants take part in defending ROS, which are deemed to be principal damage mechanism drawn forth by most environmental toxicants.[24] It is widely accepted that DEHP get into the body and produces excessive reactive oxygen species to enable increased oxidative stress in cells, which leads to cell death.[25] Thus, we thought DEHP exposure increased ROS in testes, high level of ROS generation in Sertoli cells resulted in Sertoli cells dysfunction, and eventually led to disrupt BTB integrity. Then, we gauged SOD and HO-1 expression levels in rat testis. Results manifested that SOD and HO-1 presented lower expression levels after exposure, certifying there existed oxidative stress in testicular tissues of animals after treatment. Although a lot of scholars has corroborated junctions proteins expressions decrease and oxidative stress injury after DEHP exposure, no direct evidence to certify BTB impairment caused by DEHP is through oxidative stress mechanism.

Recently, it has been demonstrated that the relative excessive accumulation of ROS could break cellular

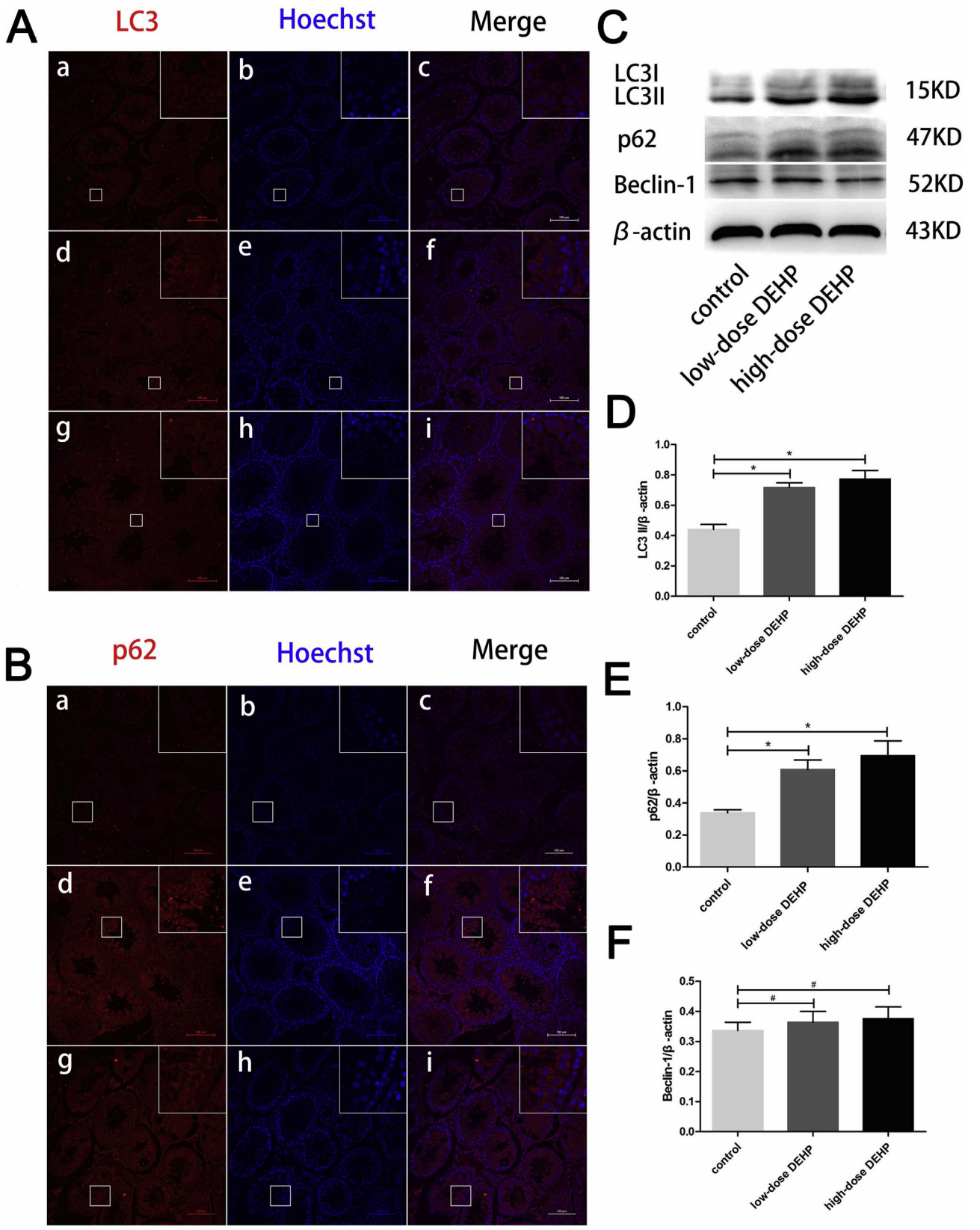

Fig. 6 DEHP exposure induces the protein expressions of LC3 and p62 in rat testes. Immunofluorescence staining showed that LC3 and p62 was amply expressed in Sertoli cell layers and spermatogenic cell layer after DEHP exposure (A and B). Western blotting analysis demonstrated that LC3 and p62 levels were upregulated both in DEHP-treated group compared with control group (D). Quantitative analysis for the immunoreactive bands in the results of western blotting analysis indicated that LC3 (E) and p62 (F) expression increased significantly in DEHP-treated group. Three sections were analyzed for each sample. * Significantly different, $p < 0.05$, # No significant different, $p > 0.05$.

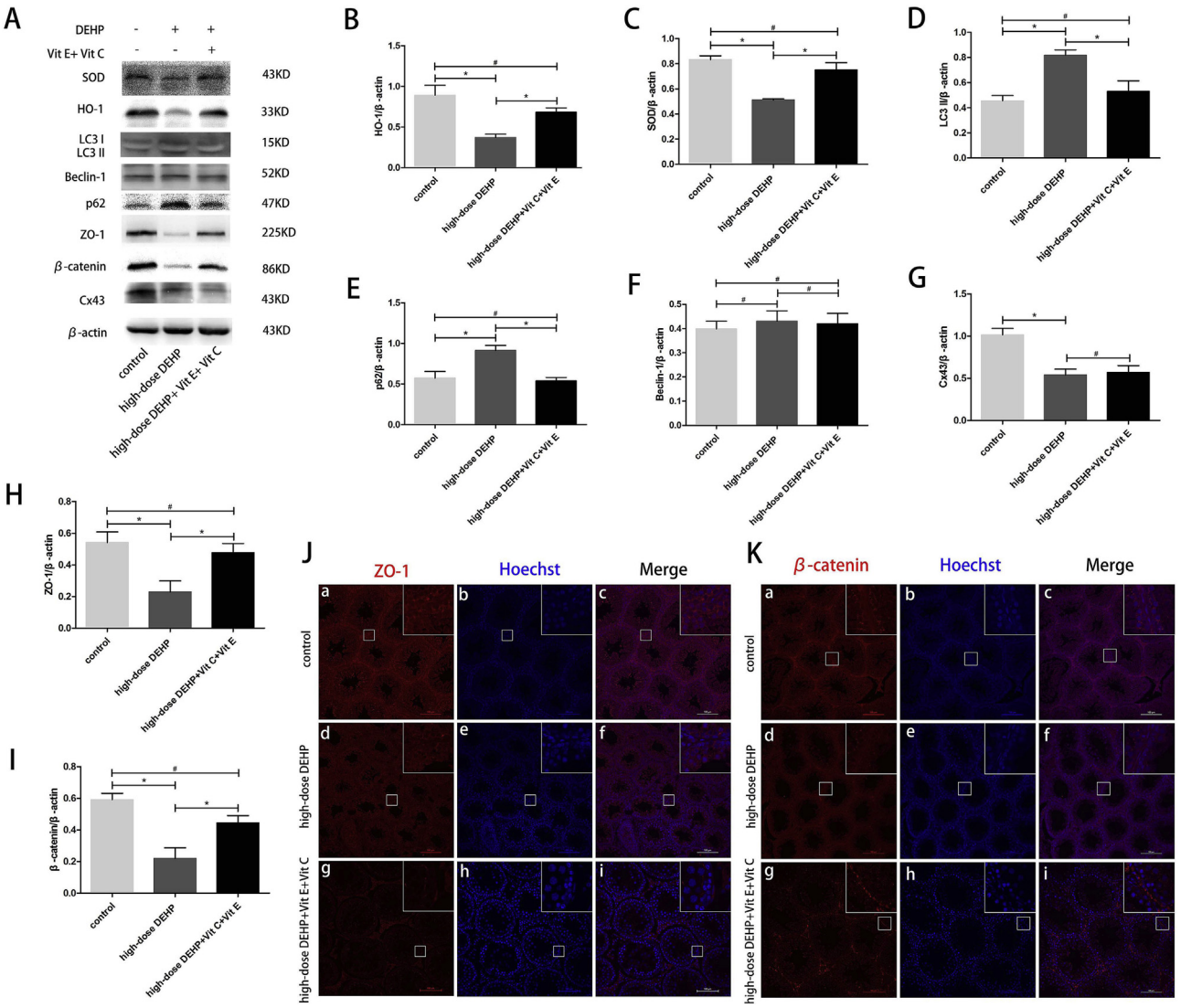

Fig. 7 The expression of SOD, HO-1, LC3II/I, p62, β-catenin, Cx43 and ZO-1 after vitamin C + E treatment. Densitometry value analysis for each independent experiment was performed at least in triplicate. Densitometry values were normalized to β-actin. *$p < 0.05$. #p> 0.05.

homeostasis, resulting in oxidative stress and mitochondrial dysfunction and induced autophagy. In this process, oxidative stress could promote the formation of autophagy.[11,12] Moreover, the function of autophagy in the context of DEHP-induced immature testicular impairment has not been explored. We performed transmission electron microscopy and increasing number of autophagosome were observed in sertoli cells and germ cells of DEHP-treated testes compared with the controls. More than 30 autophagy-related genes identified by yeast genetic analysis are involved in the autophagy and its related pathway. Among them, beclin-1 plays a role in the formation of autophagosome, p62 in the autophagic degradation process and LC3/ATG8 in the vesicular elongation of the phagophore.[1–3] We then found that the expression of LC3 was significantly increased in testes of both DEHP groups, p62, was up-regulated, whereas the expression of Beclin-1 was not altered. LC3 and p62 staining were mainly located in Sertoli cell layers and germ cells of testicular seminiferous epithelium, which suggests that the autophagy defect is caused by impaired degradation rather than formation. It may be the first study focus on the role of autophagy and the autophagy-related genes in DEHP-induced reproductive toxicity.

Vitamin C and vitamin E are reported to alleviate oxidative stress and improve fertility. However, whether these important antioxidants could work on the DEHP-induced oxidative stress is unknown. In this study, vitamins E + C supplement normalized the oxidative stress state in testes after DEHP exposure and the restored oxidative stress state rescued the autophagy defect and BTB impairment.

In summary, DEHP induces the accumulation of autophagosome and affects the integrity of BTB. The impairment of BTB finally contributes to spermatogenesis disturbance, accumulation of damaged/ unhealthy mitochondrial and lead to infertility. Moreover, up-regulated expressions of HO-1 and SOD suggest a link between processes regulating BTB integrity and autophagy via ROS in testes under DEHP

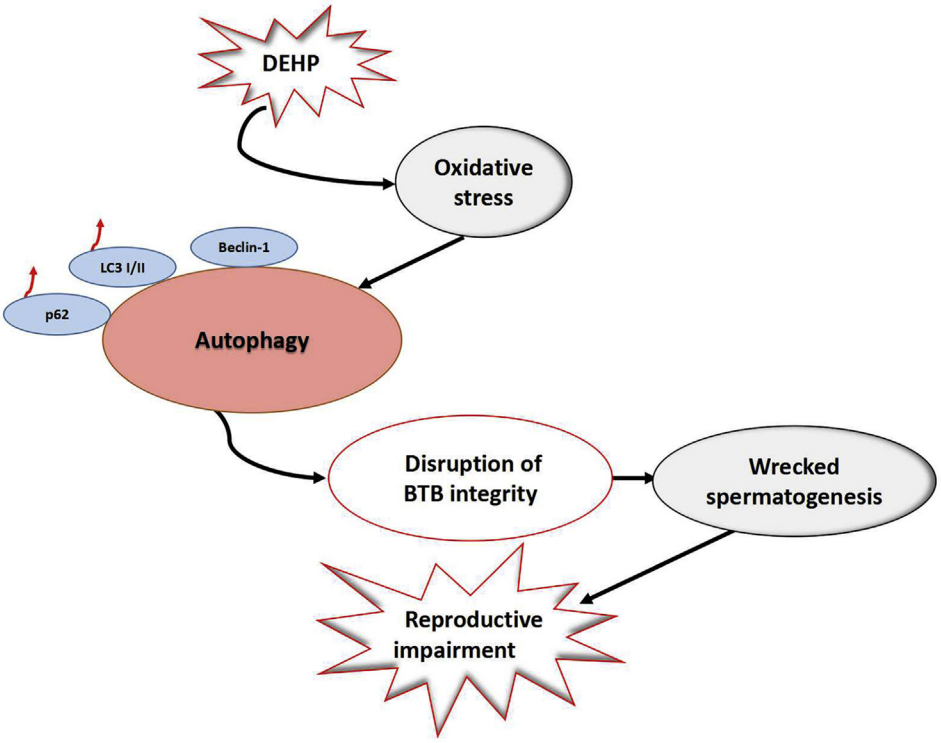

Fig. 8 Scheme of the ROS-mediated autophagy in determining BTB impairment induced by DEHP. DEHP induced autophagic accumulation via promotion of reactive oxygen species. Integrity of BTB was destroyed by activation of autophagy. Then the impairment of BTB lead to decreasing spermatogenic cells growth and finally cause infertility.

exposure (Fig. 8). Vitamins E and C could alleviate the DEHP-induced oxidative stress, rescued the autophagy defect and restored the BTB integrity. Taken together, our findings put insights into the roles of autophagy and ROS in the aggravation of testicular damage, especially the BTB damage, which could contribute to a better understanding of potential molecular mechanisms of DEHP-induced reproductive toxicity, and possibly novel potential target for preventive and/or therapeutic treatments.

Conflicts of interest

The authors declare no conflict of interests.

Acknowledgements

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