



# Histopathologic, apoptotic and autophagic, effects of prenatal bisphenol A and/or di(2-ethylhexyl) phthalate exposure on prepubertal rat testis

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## Abstract

Bisphenol A (BPA) and di(2-ethylhexyl) phthalate (DEHP) are endocrine-disrupting chemicals (EDCs) used in a wide variety of industrial products as plasticizers. Exposure to EDCs, particularly in mixtures, in prenatal and early postnatal periods may lead to unwanted effects and can cause both developmental and reproductive problems. In this study, we aimed to determine the individual and combined effects of prenatal and lactational exposure to BPA and/or DEHP on testicular histology, apoptosis, and autophagic proteins. Pregnant Sprague-Dawley rats ( $n = 3$ ) were divided into four groups (control, BPA (50 mg/kg/day), DEHP (30 mg/kg/day), and BPA (50 mg/kg/day) + DEHP (30 mg/kg/day)) and dosed by oral gavage during pregnancy and lactation. The male offspring ( $n = 6$ ) from each group were chosen randomly, and their testicular examinations were performed on the twelfth week. The results showed that fetal and neonatal exposure to BPA and DEHP could lead to significant testicular histopathological alterations and cause increases in apoptosis markers (as evidenced by increases in caspase 3 and caspase 8 levels; increased TUNEL-positive spermatogonia and TUNEL-positive testicular apoptotic cells) and autophagic proteins (as evidenced by increased LC3 and Beclin levels and decreased p62 levels) in testicular tissue. We can suggest that EDCs cause more dramatic changes in both testicular structure and cell death when there is combined exposure.

**Keywords** Phthalate · Bisphenol A · Male reproductive system · Prenatal exposure · Autophagy · Apoptosis

## Introduction

Endocrine-disrupting chemicals (EDCs) are exogenous substances. They can be found naturally or they can be man-made. These chemicals can affect the synthesis, secretion, transport, metabolism, elimination, receptor binding, and natural homeostasis of hormones, leading to reproductive and

developmental problems (Environmental Protection Agency 2017).

Synthetic EDCs consist a wide range of chemical compounds, and they are mainly used as plasticizers, industrial solvents, lubricants, or pesticides. Some EDCs, like phthalates and bisphenols, are also used in tablet coatings (Diamanti-Kandarakis et al. 2009). These chemicals can highly be present in industrial areas where they can contaminate the soil and groundwater as mixtures. Their mixtures can accumulate in the food chain, and they can cause abundant human exposure (Diamanti-Kandarakis et al. 2009; Janesick et al. 2011). Occupational exposures in various industrial fields are of particular importance and can lead to very important consequences. Their use of cosmetic products, toys, coating materials, plastic food containers, bottles, baby products, blood transfusion and dialysis bags, medical supplies, and various household chemicals are other important sources of individual exposures to EDCs (Diamanti-Kandarakis et al. 2009). Moreover, natural EDCs are present in different edible plants, like soya, garlic, parsley, or apple. However, due to short half-lives of these compounds, they do not present any harm to

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human health unless they are consumed in high amounts (Dickerson and Gore, 2007).

Bisphenol A (BPA) and phthalates are widely known EDCs to which human exposure is inevitable. Phthalates are used to increase the elasticity and softness of plastic materials. Due to their widespread use, phthalates are produced at very high quantities (David 2000; Yaghjyan et al. 2016). They are not covalently bound to plastic material and can be released to the environment, leading to abundant exposures. Humans can be exposed to these compounds through oral, inhalation, and dermal routes (Koch and Calafat 2009).

Phthalates are suggested to have anti-androgenic potential (Erkekoglu et al. 2011). Their target organ within the human body is the testis, with Sertoli and Leydig cells being the most affected cell types (Erkekoğlu et al. 2010). They are suggested to be at least one of the underlying factors in many diseases, including gynecomastia, premature thelarche, precocious puberty, and thyroid disorders (Dong et al. 2019; Liu et al. 2018; Durmaz et al. 2018). Di(2-ethylhexyl) phthalate (DEHP) is the most widely used phthalate derivative, and it is suggested to be a reprotoxicant (Erkekoğlu et al. 2010; Rowdhwal and Chen 2018; Erkekoglu et al. 2014a, b). DEHP may lead to both apoptosis and autophagy in testis, particularly in germ cells (Barakat et al. 2017).

Bisphenol A is an estrogen-like EDC and one of the most produced chemical substances in the world today. Diet and water can be the main exposure routes (Careghini et al. 2015). On the other hand, BPA can also be taken to the body through the lungs and the skin (Vandenberg et al. 2007). Moreover, occupational exposure (automobile, electronic product, sports equipment, and CD and DVD coatings) consists of another exposure route to workers (Fleisch et al. 2010; Rubin 2011). BPA is also suggested to affect male reproductive system, leading to lower sperm counts and alterations in testicular histology (Cariati et al. 2019; Minguez-Alarcon et al. 2016). This bisphenol derivative is also suggested to cause different types of programmed cell death in testis tissue (Zhang et al. 2017; Jiang et al. 2018).

Exposure to EDCs in different periods of life can cause serious adverse effects, particularly on the developing reproductive system (Diamanti-Kandarakis et al. 2009). Several researches on EDCs have shown that their effects in critical periods of development, including prenatal life and early childhood, may be more pronounced and may lead to serious consequences. However, studies that examine the effects of prenatal and lactational exposures are extremely limited (Swanson et al. 2009; Ferguson et al. 2014; de Boo and Harding 2006). Prenatal combined exposure to DEHP and BPA (0.1, 1, and 10 mg/kg/day, both) caused a dose-dependent decrease in sex ratio (male/female) in mice at post-natal day (PND) 15. In addition, a dose-dependent decrease in testicular weight, sperm count, testicular anti-Mullerian hormone (AMH), follicle-stimulating hormone (FSH),

progesterone, and testosterone. These unwanted effects stayed persistent even at PND 42 in the sexually mature animals (Xi et al. 2011a, b). DEHP exposure was also suggested to inhibit luteal function via dysregulation of platelet endothelial cell adhesion molecule (CD31) and prostaglandin F2 $\alpha$  in pregnant mice (Guo et al. 2015).

There are several studies in literature that show the individual effects of phthalates or BPA on a developing male reproductive system. However, studies on combined exposures are limited, and they do not present a comprehensive evaluation. It is anticipated that the adverse effects after combined exposure may have exacerbated consequences. Concerning all the available data, the present study aimed to evaluate the effects of prenatal and lactational exposure to BPA and/or DEHP on prepubertal rat testis. Histopathologic and apoptotic changes in testicular tissue were investigated, and autophagy marker proteins were also evaluated.

## Materials and methods

### Chemicals and reagents

Di(2-ethylhexyl) phthalate and BPA were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Commercial caspase 3 and caspase 8 kits and “In Situ Cell Death Detection Kit” were purchased from MyBioSource (San Diego, CA) and Roche (Mannheim, Germany), respectively. Commercial ELISA kits for detection autophagic biomarkers p62, human microtubule-associated proteins 1A/1B light chain 3B (MAP1LC3B), and Beclin-1 were purchased from Enzo Biochem (New York, NY), MyBioSource (San Diego, CA), and Aviva Systems Biology (San Diego, CA), respectively.

### Animals and dosing regimen

Di(2-ethylhexyl) phthalate was prepared in corn oil. BPA was first dissolved in 1 mL 96% ethanol (6.25 mg/mL) and then diluted with corn oil. The doses were selected as lowest observed adverse effect levels (LOAEL) on the male reproductive system for DEHP (30 mg/kg/day) and for BPA (50 mg/kg/day) (Agency for Toxic Substances and Disease Registry 2002; Shelby 2006; Tomza-Marciniak et al. 2018; European Food Safety Authority 2007).

All animals were supplied from Gazi University Experimental Animals Laboratory (Ankara, Turkey). Pregnant Sprague-Dawley rats were divided randomly to four groups ( $n = 3/\text{group}$ ): Controls received corn oil; BPA group received 50 mg/kg/day BPA; DEHP group received 30 mg/kg/day DEHP; and BPA + DEHP group received 50 mg/kg/day BPA and 30 mg/kg/day DEHP through 6–21 gestational days and lactation period by intra-gastric lavage. Male offspring ( $n = 6/\text{group}$ ) from each mother were fed until the end of the

twelfth postnatal week. They were then weighed and euthanized (cardiac exsanguination under deep anesthesia) and the testes were removed. The testes were weighed and relative testis weights were later calculated. The animals were treated humanely and with regard for alleviation of suffering, and the study was approved by Gazi University Ethical Committee (G.U.ET-15.066).

### Body and organ weights

On the first day of birth, both female and male offspring were weighed. At the end of the lactation period, the male offspring were randomly selected, and the study groups were formed. The male pups were weighed again.

### Histopathology and electron microscopy

The left testis was divided into two pieces. One piece was used for histopathological evaluation, and the other piece was used for electron microscopy (EM). The first piece was fixed in Bouin's fixation solution for 18 h, and tissue samples were taken on a Leica TP 1020 light microscope tissue processor (Wetzlar, Germany). In order to provide dehydration, samples were passed through graded alcohols on the tissue processor. All specimens were embedded in paraffin, and 5- $\mu$ m sections were cut from the obtained paraffin blocks. These sections were left overnight at 60 °C and were then deparaffinized with xylene. The dehydration step was repeated. Sections were stained with hematoxylin and eosin. The sections were examined under a Leica DM6000B microscope (Wetzlar, Germany) and images were taken by a Leica DC490 digital camera (Wetzlar, Germany) (Saadeldin et al. 2011).

The second piece of the fresh tissue sample was fixed in 2.5% glutaraldehyde solution in phosphate buffer, (pH 7.4, 4 h) and was post-fixed in 1% osmium tetroxide solution (in 0.1 M phosphate buffer) for 1 h. Later, tissue samples were washed with phosphate buffer and samples were dehydrated in a graded series of alcohols. Samples were treated with propylene oxide and embedded in Araldite/Epon812, and heat polymerization was performed. Sections were cut using a microtome (Leica Ultracut R); semi-thin sections were stained with methylene blue–azure II and examined using an electron microscope (Leica DM6000B, Wetzlar, Germany) with a Leica DC490 digital camera. Ultrathin sections were double-stained with uranyl acetate and lead citrate (Leica EM AC20).

### TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay has been designed to detect apoptotic cells that undergo extensive DNA degradation during the late stages of apoptosis. The method is based on the ability of terminal deoxynucleotidyl transferase (TdT) to label blunt

ends of double-stranded DNA breaks independent of a template. Apoptotic cells in testis tissues were identified by a TUNEL assay using In Situ Cell Death Detection Kit-POD according to manufacturers' instructions. Briefly, paraffin sections of testis (5  $\mu$ m in thickness) were deparaffinized, rehydrated, and washed twice in PBS for 5 min. Then the slides were incubated with 3% H<sub>2</sub>O<sub>2</sub> for 10 min in order to block endogenous peroxidase activity. After washing in PBS three times for 5 min, the slides were incubated with permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 8 min at 4 °C and later washed twice with PBS for 5 min. The labeling reaction was performed by using 50  $\mu$ l TUNEL reagent for each sample, except the negative control, in which reagent without enzyme was added and incubated for 1 h at 37 °C. Following PBS washings, the slides were incubated with converter reagent for 30 min at 37 °C. After washing, the slides were incubated with 3,3'-diaminobenzidine (DAB) solution for 10 min for labeling of cells containing DNA strand breaks. In tissue samples, TUNEL-positive stained cells were counted in 20 seminiferous tubules with light microscope (DM6000B, Wetzlar, Germany) at  $\times$  400 magnification, and the average number of apoptotic spermatogenic cells was determined for the study groups.

### Testis tissue preparation for the determination of apoptotic and autophagic proteins

The right testes were removed and washed in saline and deionized water. The testes were frozen immediately in liquid nitrogen and kept at – 80 °C. For the determination of apoptotic and autophagic proteins, testes were homogenized in a homogenization buffer (10 mM Tris/1 mM DTPA/1 mM PMSF) by using a Teflon-glass homogenizer. The homogenates were then centrifuged at 2000 $\times$ g at 4 °C for 10 min. The supernatants were used in the determination of apoptotic and autophagic proteins.

### Caspase 3 and caspase 8 levels

Both caspase 3 and caspase 8 levels were measured by commercial kits using sandwich ELISA method. The well plates within the kits were pre-coated with either caspase 3 or caspase 8 antibodies. The biotin conjugated anti-CASP3 or anti-CASP8 antibodies were used as “detection antibodies.” The standards or test samples were added to wells. Plates were incubated at 37 °C for 90 min, later discarded, and washed, and biotin-conjugated detection antibodies were added. The plates were incubated at 37 °C for 60 min and washed, and HRP-streptavidin conjugate (SABC) was later added. The plates were again incubated at 37°C for 30 min and washed. 3',5,5'-Tetramethylbenzidine (TMB) substrate was added, and plates were incubated in the dark at 37°C for 30 min for color development. Later, stop solution was added, and the

absorbance was read at 450 nm. Both of the kits had high sensitivity and specificity to caspase 3 or caspase 8.

### Autophagy biomarkers

Testicular p62, human microtubule-associated proteins 1A/1B light chain 3B (MAP1LC3B), and Beclin-1 levels were measured as autophagy biomarkers. These biomarkers were measured with commercial ELISA kits based on standard sandwich ELISA method.

### Statistical analysis

The results were expressed as mean ± standard deviation (SD). The differences among the groups were evaluated with Kruskal–Wallis one-way analysis of variance, followed by Mann–Whitney *U* test using a Statistical Package for Social Sciences Program (SPSS) version 17.0. *p* values < 0.05 were considered statistically significant.

## Results

### Body weights

The average body weights of the pups on the first day of birth are given in Table 1. The birth weights of the pups in the DEHP group were significantly higher than that of the control (8%) and BPA (10%) groups. In addition, the birth weights of the BPA + DEHP group were found to be higher than that of the control (12%) and DEHP (4%) groups (*p* < 0.05, both).

At the end of the lactation period, the mean body weight of male pups was significantly higher in DEHP (13%) and BPA + DEHP (6%) groups compared with that of the control group. The mean body weights of male offspring in BPA (19%) group were significantly decreased vs. that of the control (Table 1).

**Table 1** Mean birth weight

	Birth weight (g)	Body weight (g) End of lactation period
Control	4.90 ± 0.5 <sup>a</sup>	50.87 ± 2.9 <sup>a</sup>
BPA	4.79 ± 0.8 <sup>a</sup>	41.17 ± 2.6 <sup>b</sup>
DEHP	5.29 ± 0.2 <sup>b</sup>	57.33 ± 2.7 <sup>c</sup>
BPA + DEHP	5.48 ± 0.2 <sup>c</sup>	54.08 ± 1.5 <sup>d</sup>

All results were given as mean ± SD. Bars that do not share same letters (superscripts) are significantly different from each other (*p* < 0.05). Nineteen offspring in the control group, 29 offspring in BPA group, 12 offspring in the DEHP group, and 18 offspring in the BPA + DEHP group

### Testis and epididymis weights

The mean testis and epididymis, and relative testis and epididymis weights of the study groups were given in Table 2. There were no significant differences in testis and epididymis weights between the study groups and control; however, the DEHP group had significantly higher testis weights vs. the BPA group.

Relative testis weights of all the study groups were not different than that of the control; however, the DEHP group had significantly higher relative testis weights vs. the BPA group. On the other hand, relative epididymis weights of all the study groups were higher than that of the control (*p* < 0.05, all; 5% in BPA, 18% in DEHP, and 23% in BPA + DEHP).

### Histopathology

In the control group, seminiferous tubule sections and interstitial tissue between seminiferous tubules were in normal morphological organization within the testis sections surrounded by a connective tissue called “tunica albuginea” externally. The seminiferous tubules containing myoid cells from the outside were wrapped with a fine connective tissue called “tunica propria.” There were not any degenerations in the spermatogenic cells and Sertoli cells. No abnormalities were observed in spermatogenesis and spermiogenesis (Fig. 1 a and b).

In BPA group, the integrity of the epithelium was impaired in some seminiferous tubules. Primer spermatocytes were separated from the basal lamina in some regions (Fig. 1 c). Spermatogonia (located immediately adjacent to Sertoli cells in the basal lamina layer of the seminiferous tubules) were not observed in some areas of the tubules (Fig. 1 d).

Significant segregations were observed in the samples of the DEHP group indicating that the intercellular connections between the Sertoli cells and the spermatogonia were impaired on the basis of seminiferous tubules. Separations were also observed among the cells of the spermatogenic series that were differentiating on the walls of the seminiferous tubules. In the lumen of some seminiferous tubules, round-shaped spermatogenic cells were spilled, and cell debris was observed in the lumen (Fig. 1 e).

In BPA + DEHP group, testis tissues maintained their presence in the interstitial tissue between the seminiferous tubules by preserving the normal histological structure of the Leydig cells. In this group, large gaps were observed between the cells of the spermatogenic series in the affected seminiferous tubules (Fig. 1 f). In addition, we observed that the loss of the cells in the advanced differentiation stages caused a decrease in the thickness of epithelia (Fig. 1 g). Spermatogenesis and spermiogenesis were both impaired (Fig. 1 h).

**Table 2** Testis and epididymis weights

	Testis weights (mg/g)	Epididymis weights (mg/g)	Relative testis weights (mg/g)	Relative epididymis weights (mg/g)
Control ( <i>n</i> = 6)	1.45 ± 0.21 <sup>ab</sup>	0.53 ± 0.05 <sup>a</sup>	0.0060 ± 0.0009 <sup>ab</sup>	0.0022 ± 0.0002 <sup>a</sup>
BPA ( <i>n</i> = 6)	1.23 ± 0.13 <sup>a</sup>	0.50 ± 0.02 <sup>a</sup>	0.0058 ± 0.0007 <sup>a</sup>	0.0023 ± 0.0001 <sup>ab</sup>
DEHP ( <i>n</i> = 6)	1.41 ± 0.09 <sup>b</sup>	0.53 ± 0.04 <sup>a</sup>	0.0071 ± 0.0006 <sup>b</sup>	0.0026 ± 0.0002 <sup>bc</sup>
BPA + DEHP ( <i>n</i> = 6)	1.45 ± 0.18 <sup>ab</sup>	0.59 ± 0.13 <sup>a</sup>	0.0069 ± 0.0014 <sup>ab</sup>	0.0027 ± 0.0002 <sup>c</sup>

All results were given as mean ± SD. Bars that do not share same letters (superscripts) are significantly different from each other ( $p < 0.05$ ). Six animals per group

## Electron microscopy

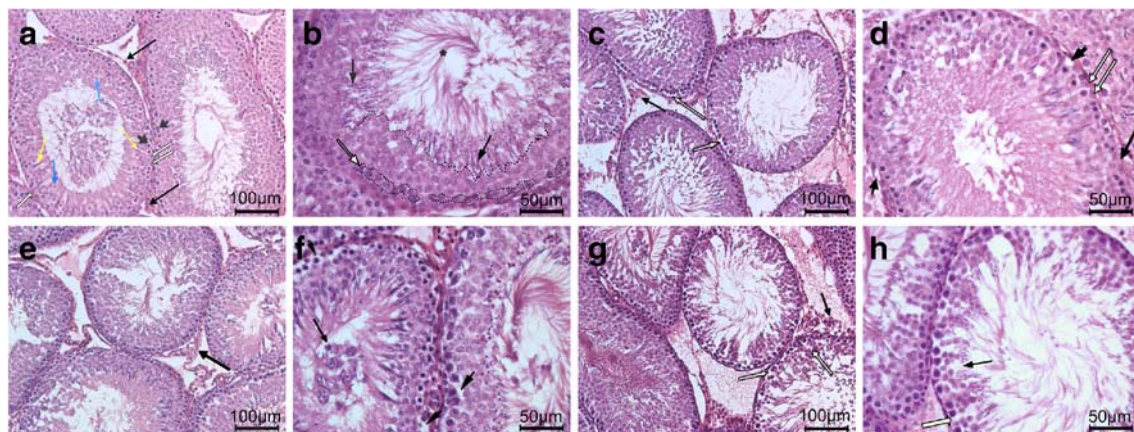
Sertoli cells with indented nucleus lying over the basement membrane were observed in the control group. Spermatogonium and spermatocytes belonging to different stages of maturation, such as primary spermatocytes and spermatids, were observed in the neighboring region of Sertoli cells (Fig. 2 a).

In BPA group, spermatogenesis was disrupted, and degenerated cells with condensed nuclei were observed instead of spermatogonium and spermatocytes between the

Sertoli cells in the basal and luminal compartment of affected seminiferous tubules. The cytoplasmic remnants of spermatogenic cells were observed in the wall of seminiferous tubules (Fig. 2 b).

Spermatogenic cells and Sertoli cells were present in the DEHP group. Lipid droplets, lysosomes, and vacuoles were observed in the cytoplasm of Sertoli cells in the DEHP group (Fig. 2 c).

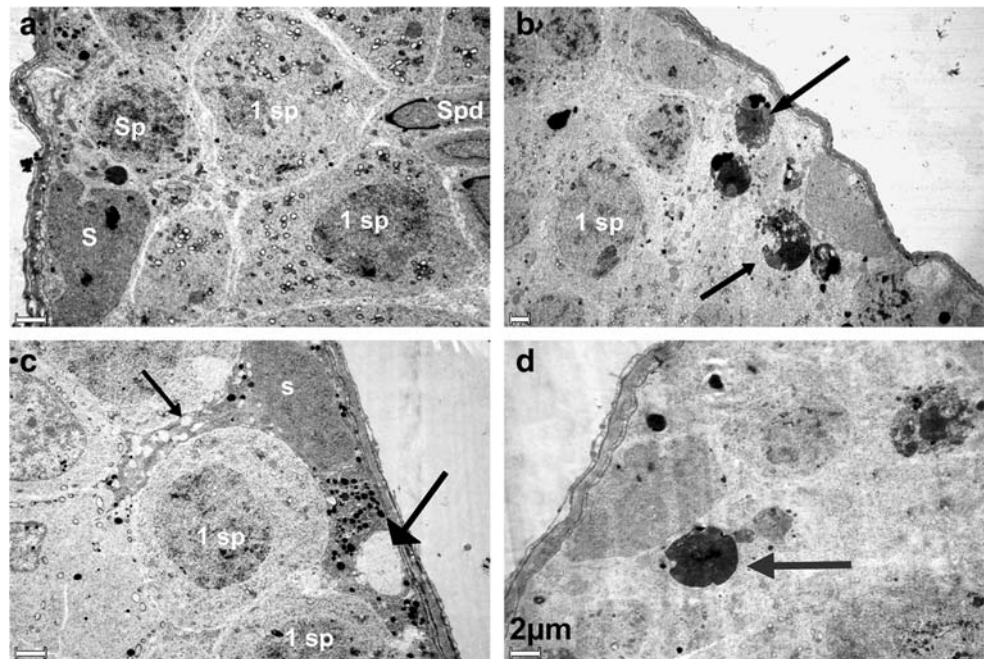
Spermatogenesis was more affected in the BPA + DEHP group when compared with that of the BPA or DEHP groups. Degeneration in spermatogenesis with pyknosis and



**Fig. 1** Representative histological sections of the testis of rats (H&E). **a** Light micrograph of the seminiferous tubules from the control group. Leydig cells (black arrow) between seminiferous tubules, Sertoli cells (white arrow), and the cells of spermatogenic series (primary spermatocytes (arrow head), early spermatid (blue arrow), and late spermatid (yellow arrow)) in the seminiferous tubule wall are seen with normal histological appearance (Hematoxylin-Eosin × 200). **b** Light micrograph of seminiferous tubule from the control group. Dashed line separates the spermatogenic cells at different stages. Late spermatids with elongated and condensed nucleus (black arrow) and primary spermatocytes (white arrow) are observed in the process of spermatogenesis in the seminiferous tubule wall, and tails of the spermium (asterisk) are seen in the lumen at higher magnification. (Hematoxylin-Eosin × 400). **c** Light micrograph of seminiferous tubules from the BPA group. Leydig cells (black arrow) among the seminiferous tubules are observed in normal histological appearance. The spermatogenic cells (white arrow) are separated from the basal lamina in some of the areas of the seminiferous tubule wall (Hematoxylin-Eosin × 200). **d** Light micrograph of seminiferous tubule from the BPA group. Sertoli cells (white arrow) are seen on the basal lamina in the wall of the seminiferous tubule. Occasionally, the loss of spermatogonium (black arrow)

and empty spaces (arrowhead) are observed near these cells at higher magnification. (Hematoxylin-Eosin × 400). **e** Light micrograph of seminiferous tubules from the DEHP group. In interstitial area Leydig cells (arrow), flat nucleated fibroblasts, and collagen fibers are seen in the DEHP-exposed group. Separation was observed between the cells in the wall of the seminiferous tubule (Hematoxylin-Eosin × 200). **f** Light micrograph of seminiferous tubule from the DEHP group. Separation (arrowhead) is seen between primary spermatocytes and also between primary spermatocytes and advanced spermatogenic serial cells. Cell debris (arrow) is observed in the lumen of the seminiferous tubule in the same group (Hematoxylin-Eosin × 200). **g** Light micrograph of seminiferous tubules from the BPA + DEHP group. Large gaps (white arrow) were observed between the spermatogenic cells in the cross sections of the samples belonging to the group exposed BPA + DEHP. Leydig cells (black arrow) are seen in the interstitial area (Hematoxylin-Eosin × 200). **h** Light micrograph of seminiferous tubule from the BPA + DEHP group. Gaps between the primer spermatocytes and the basal lamina and neighboring cells (white arrow) were observed at higher magnification. The loss of cells in the advanced stage of differentiation in the seminiferous tubule epithelium (black arrow) was observed (Hematoxylin-Eosin × 400)

**Fig. 2** Transmission electron micrographs of seminiferous tubules of experimental groups. **a** Sertoli cells with indented nucleus and different stages of spermatogonial cells in the surrounding in control. **b** Degenerated cells (arrow) in BPA group. **c** Lipid droplets, lysosomes, and vacuoles (arrow) in the cytoplasm of Sertoli cells and two primary spermatocyte connected by cytoplasmic bridges in DEHP group. **d** Degeneration in spermatogenesis with pyknotic spermatogenic cells (arrow) in BPA + DEHP; S: Sertoli cell, Sp: spermatogonium, 1 sp: primary spermatocyte, Spd: spermatid, Scale bar: 2  $\mu$ m



chromatin condensation in the spermatogenic cells was observed, and the nuclei of spermatogenic cells were faded and could not be distinguished in this group (Fig. 2 d).

**TUNEL assay**

The number of apoptotic spermatogenic cells (Table 3) was increased statistically in BPA, DEHP, and BPA + DEHP treated groups compared to that of the control ( $p < 0.05$ ). The mean number of apoptotic cells was the highest in the BPA + DEHP group and was statistically higher than both DEHP and BPA groups ( $p < 0.05$ ). The images of the seminiferous tubule sections are presented in Fig. 3 a, b, c, and d. Increased TUNEL-positive spermatogonia and TUNEL-positive apoptotic cells were detected in all study groups compared with that of the control group.

**Table 3** Testis tissue apoptotic cell count

	Apoptotic cell count
Control	13.29 ± 3.52 <sup>a</sup>
BPA	32.07 ± 5.15 <sup>b</sup>
DEHP	27.18 ± 2.77 <sup>b</sup>
BPA + DEHP	41.12 ± 3.11 <sup>c</sup>

All results were given as mean ± SEM. Columns that do not share same letters (superscripts) are significantly different from each other ( $p < 0.05$ ). Six animals per group

**Caspase 3 and caspase 8 levels**

There were no statistically significant differences between testicular caspase 3 levels (Fig. 4 a) due to high standard deviation within the groups ( $p < 0.05$ ). However, caspase 3 levels of BPA (29.5%), DEHP (23.71%), and BPA + DEHP groups (56.5%) were found to be higher than that of the control. In DEHP (15.9%) and BPA + DEHP (12.8%) groups, caspase-8 levels (Fig. 4 b) were found significantly higher than that of the control group ( $p < 0.05$ ).

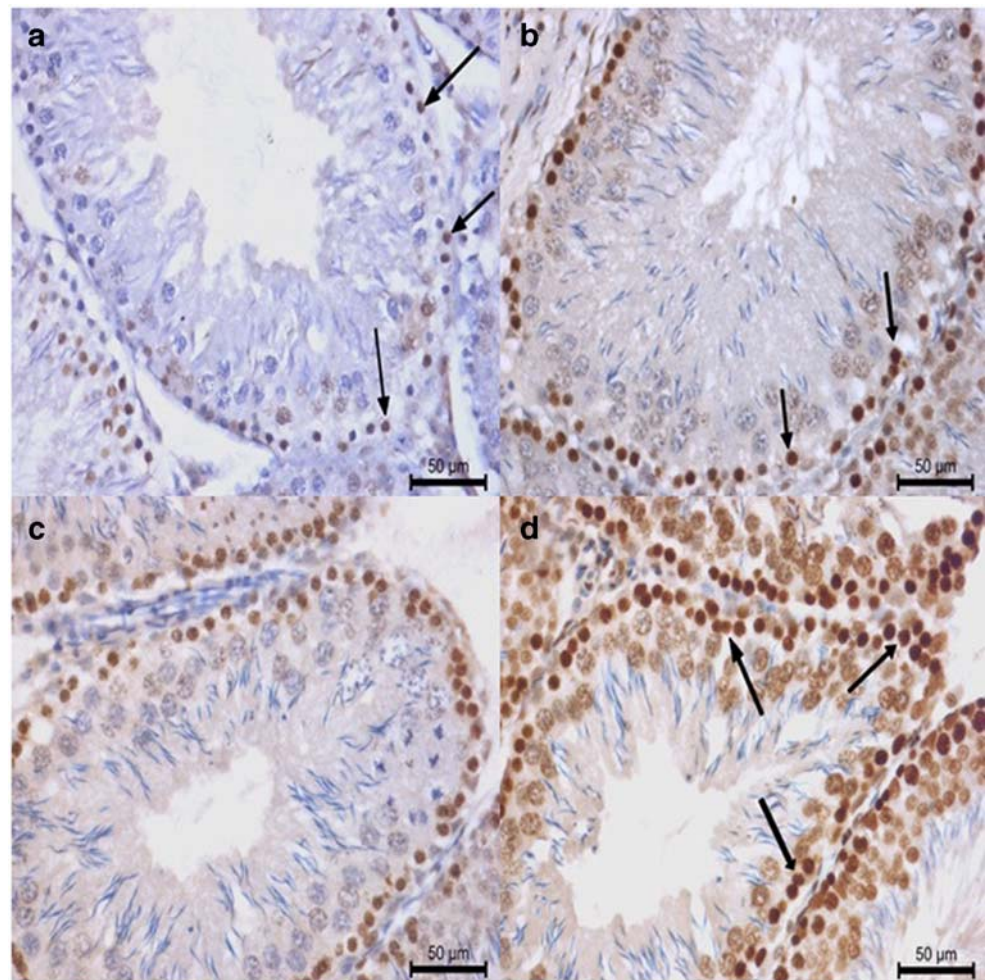
**Autophagy biomarkers**

LC3 levels (Fig. 5 a) of the testicular tissue were found to be increased with BPA (63%,  $p > 0.05$ ), DEHP (81%,  $p < 0.05$ ), and BPA + DEHP (72% increase,  $p < 0.05$ ) groups vs. that of the control. Testis Beclin levels (Fig. 5 b) increased in BPA (10%,  $p > 0.05$ ), DEHP (61%,  $p < 0.05$ ), and BPA + DEHP (40%,  $p < 0.05$ ) groups when compared with that of the control. Testis p62 levels (Fig. 5 c) were decreased in BPA (34%,  $p > 0.05$ ), DEHP (46%,  $p > 0.05$ ), and BPA + DEHP groups (91%,  $p < 0.05$ ) were lower than that of the control group. The insignificant changes in testis p62 levels in BPA and DEHP groups were due to the high variation within these study groups.

**Discussion**

Several studies have shown that the most significant toxic effects of phthalates are on the male reproductive system

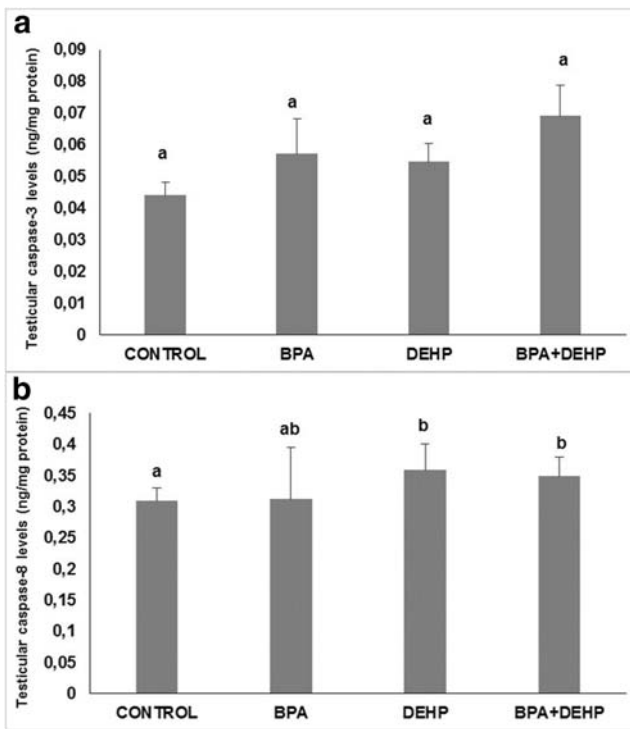
**Fig. 3** The representative images showing the TUNEL labeling of the seminiferous tubule sections of experimental groups. **a** TUNEL-positive apoptotic cells were observed in brown (arrow) in the control group. ( $\times 400$ ). **b** The number of TUNEL-positive spermatogonium (arrow) was more in BPA than that of the control group ( $\times 400$ ). **c** An increased number of TUNEL-positive apoptotic spermatogonial cells were observed in DEHP compared with that of the control group ( $\times 400$ ). **d** Intensive TUNEL-positive apoptotic cells were observed in the BPA + DEHP group. ( $\times 400$ )



and normal developmental processes. Phthalates were suggested to cause decreases in testosterone levels and anogenital distance. Moreover, other problems like testicular anomalies, undescended testis, and testicular atrophy can arise from exposure to DEHP at early stages of life (Gray et al. 2006; Talsness et al. 2009; Wei et al. 2012). Studies on the effects of prenatal and postnatal DEHP exposure showed that this particular phthalate derivative can lead to male reproductive system disorders, intrauterine deaths, and testicular atrophy (Carbone et al. 2010; Saillenfait et al. 2011). Exposure to DEHP 0, 0.05, 5, and 500 mg/kg/day (in diet, from gestational day 1 until the end of lactation) caused long-term pituitary-gonadal axis disruption in male and female mice offspring. DEHP caused a dose-dependent decrease in blastocyst rate and cleavage rate of males and decreased both sperm count and live sperm in the viable offspring along with dose-dependent decreases in CYP19A1 protein levels (Pocar et al. 2012). Gestational exposure to BPA, on the other hand, is suggested to cause perturbed reproduction. However, a clear consensus is not yet reached whether prenatal BPA exposure in humans results in direct adverse effects on male genital development and reproduction or not (Williams et al. 2014).

Although there are many regulations in order to reduce human exposure and environmental contamination of both phthalates and bisphenols, humans are still highly exposed to these chemicals starting from early stages of life (Barrios-Estrada et al. 2018; Bilal et al. 2019). Moreover, several antioxidants have been investigated in order to mitigate the toxic effects of these EDCs in different systems, and some of these substances were found to be protective against their toxicity (Erkekoglu et al. 2010, 2014a, b; Mohammed et al. 2020).

In vitro, in vivo, and human studies have shown that BPA may also have a wide variety of adverse effects on human health. The major effects are suggested to be reproductive and developmental problems, due to estrogenic effects of BPA. In addition, BPA was proposed to cause metabolic diseases (diabetes and obesity), endocrine disorders (premature thelarche, puberty precoc), thyroid hormone disorders, and oxidative stress in different organs. BPA may also have teratogenic effects like low birth weight, brain damage, and behavior disorders, and BPA exposure may be one of the underlying causes of autism (Rochester 2013; Muhamad et al. 2016; Guida et al. 2015; Stein et al. 2015; Chitra et al. 2003; Bindhumol et al. 2003; Kabuto et al. 2004; Meeker et al.

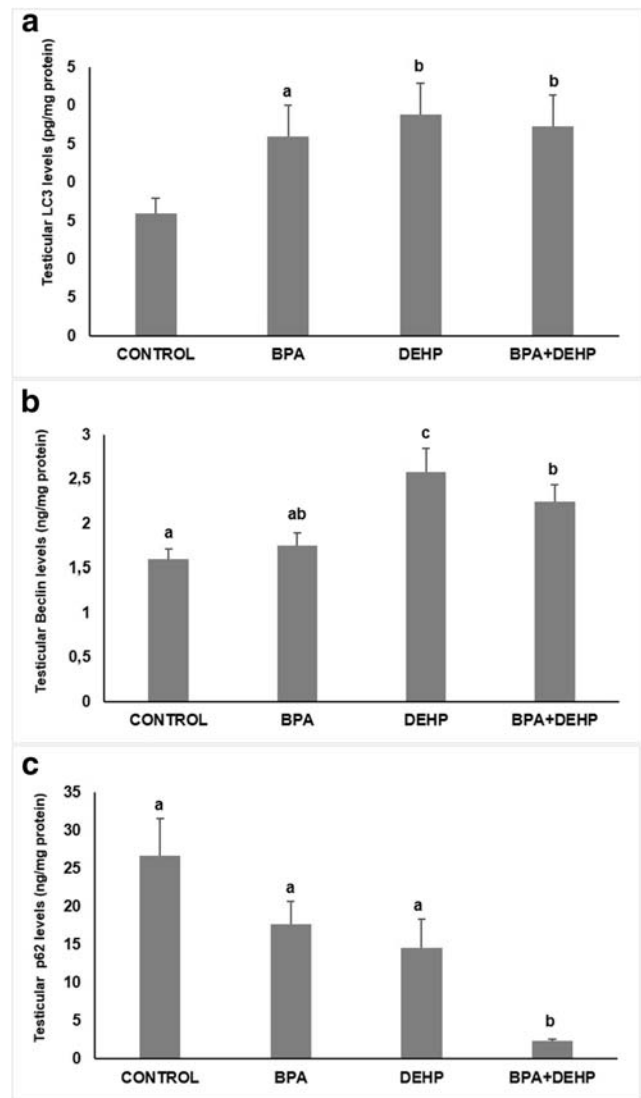


**Fig. 4** **a** Testis tissue caspase 3 levels. **b** Testis tissue caspase 8 levels. All results were given as mean ± SEM. <sup>a,b</sup>Bars that do not share same letters (superscripts) are significantly different from each other ( $p < 0.05$ )

2010; Melzer et al. 2011; Lassen et al. 2014; Gramec Skledar and Peterlin Masic 2016; Usman and Ahmad 2016).

In the histopathological studies, the integrity of the epithelium was impaired in some seminiferous tubules, and the primary spermatocytes were separated from basal lamina in some regions in BPA-exposed animals. Spermatogonia, which are immediately adjacent to Sertoli cells in the normal structure basal compartment of seminiferous tubules, were not found in some areas of the seminiferous tubules in BPA group. Several studies have shown that prenatal BPA exposure can cause various disorders in the rodent testis during adulthood. These conditions were suggested to be impaired spermatogenesis, the presence of immature spermatogenic cells in the testis tubule lumen, the presence of large gaps between Sertoli cells, and tubular lumen expansions (Kass et al. 2015; Ma et al. 2017). These results are consistent with our histopathology results due to prenatal BPA exposure. In another study conducted in newborn male rats, BPA was administered subcutaneously (0.01, 0.1, and 5 mg/kg bw), and spermatogenesis deteriorated due to BPA exposure (Xie et al. 2016). On the contrary, there are also studies that propose that maternal BPA exposure does not cause histopathologic changes in the testicular tissue of the offspring (Xi et al. 2011a, b; LaRocca et al. 2011).

In the DEHP group, there were clear separations between the Sertoli cells and the spermatogonia, indicating that the



**Fig. 5** **a** Testis tissue LC3 levels. **b** Testis tissue BECLIN levels. **c** Testis tissue p62 levels. All results were given as mean ± SEM. <sup>a,b,c</sup>Bars that do not share same letters (superscripts) are significantly different from each other ( $p < 0.05$ )

intercellular connections were disrupted in the basal lamina of the seminiferous tubules. Separations were also observed among the cells of the spermatogenic series that were differentiating on the wall of the seminiferous tubules. In the lumen of some seminiferous tubules, round-shaped spermatogenic cells of the early phase of spermium differentiation were spilled, and cell debris was observed in the lumen. Akingbemi et al. (2001) suggested that the effects of DEHP on Leydig cells were reversible even if rats were exposed to this particular phthalate for a long period of time. There are also several studies showing that maternal DEHP exposure causes histopathologic changes in testis tissue and deterioration in spermatogenesis and that these changes can be transmitted to later generations (Borch et al. 2006; Christiansen



et al. 2010; Li et al. 2013; Andrade et al. 2006; Jones et al. 2015; Doyle et al. 2013). In a previously study, Erkekoglu et al. (2011) aimed to detect toxic effects of DEHP on rat male reproductive system. Adult male Sprague-Dawley rats were separated into 6 groups. DEHP-treated groups received 1 g DEHP/kg dose by gavage during the last 10 days of the feeding period. Reproductive toxicity and anti-androgenic effects of DEHP were evidenced by disturbed testicular histology and spermatogenesis.

In the combined exposure group, we observed that the normal histological structure of Leydig cells in interstitial tissue between seminiferous tubules was preserved. However, large vacuoles were present between the cells of the spermatogenic series in the affected seminiferous tubules. In addition, the thickness of the epithelium decreased with the loss of advanced cells of the differentiation in seminiferous tubule epithelium along with impairments in spermatogenesis and spermiogenesis. Therefore, due to histopathologic changes in seminiferous tubules and Sertoli cells, we may suggest that the number and morphology of the sperms might decrease. There is one study in literature that suggests DEHP or dibutyl phthalate (DBP) exposures or DEHP plus DBP exposures may lead to histopathological alterations in rat testis. However, there is not any study in literature that evaluates the testicular effects of prenatal BPA plus DEHP exposure (Howdeshell et al. 2007; Manikkam et al. 2013). We can also propose that early-life combined exposure to BPA and DEHP might impair male infertility.

Our electron microscopy results showed that both BPA and DEHP exposure caused deterioration in testicular tissue. Spermatogenesis was affected within all of the study groups, particularly in the combined exposure group. In a recent study by Qin et al. (2018), 5-week-old Sprague-Dawley rats were exposed to different doses (0 mg/kg, 100 mg/kg, 500 mg/kg, and 1500 mg/kg) of DEHP for 6 weeks. In the high dose (1500 mg/kg) group, DEHP induced the formation of vacuoles in the Sertoli cells and abnormal proliferation of Leydig cells. Moreover, DEHP also caused the impairment of different stages of spermatogenic cells in seminiferous tubules. In another study, pregnant rats were exposed to DEHP (125, 250, and 500 mg/kg bw/day) orally between gestational days 7 and 18. The electron microscopy results revealed that histopathological changes were observed in fetal testis at high doses but not in the lowest dose. However, the postnatal observations revealed that all of the study groups showed testicular abnormalities after 5 and 10 weeks following birth (Shirota et al. 2005).

In a study by Vijaykumar et al. (2017), the researchers evaluated the histopathological effects of oral BPA administration at different doses (2.5, 12.5, and 25  $\mu$ g/kg bw/day, for 70 days) on testicular tissue in 16 adult male monkeys. Histopathological evaluation and transmission electron microscopy results showed that sloughing of germ cells into

the lumen was present along with the increases in interstitial space and vacuolation of Sertoli cell cytoplasm. Moreover, several degenerative effects on the basement membrane, Sertoli cells, Leydig cells, and other developing germ cells were observed in the 12.5 and 25  $\mu$ g/kg bw/day groups when compared with that of the control.

Apoptosis is a type of programmed cell death in which a hemostatic mechanism works for the maintenance of healthy cell populations during different stages of life. All of the exposure groups (BPA, DEHP, and BPA plus DEHP) showed a statistically significant increase in apoptotic spermatogenic cell count compared with that of the control ( $p < 0.05$ ). The mean number of apoptotic cells was the highest in the BPA plus DEHP-exposed group, and the number of apoptotic cells in the combined exposure group was significantly higher than both DEHP- and BPA-exposed groups. There are studies in literature that suggest exposure to BPA in prenatal period caused increases in apoptosis in testicular tissue (the doses and treatment periods of these studies were given above). However, some studies suggest the contrary (Ma et al. 2017; LaRocca et al. 2011). Manikkam et al. (2013) found that maternal exposure (gestational day 8–14) to high-dose mixture of BPA (50 mg/kg bw/day), DEHP (750 mg/kg bw/day), and DBP (66 mg/kg bw/day) and lower-dose mixture (one-half dose as plastics group) caused significant increases in the number of apoptotic spermatogenic cells in the F3 generation; however, apoptotic cell number decreased in F1 generation. In another study, BPA administration to adult Sprague-Dawley rats resulted in an increase in the number of apoptotic cells in the testicular tissue vs. control at doses of 100 and 200 mg/kg bw/day for 4 weeks, while no significant change was observed in the dose of 50 mg/kg bw/day (Wang et al. 2014). In another study, BPA was administered subcutaneously (0.01, 0.1, and 5 mg/kg) to neonatal male rats on postnatal day (PND) 1 to 21. Significant increases in germ cell apoptosis was observed compared the control (Xie et al. 2016). Moreover, BPA exposure has also been shown to induce apoptosis in primary culture of Sertoli cells which were isolated from 18- to 22-day-old Sprague-Dawley rats at low doses (50, 70  $\mu$ M) in vitro (Qi et al. 2014). In a study by Murphy et al. (2014), MEHP was administered to young rats (PND 21, 28, and 35) orally. Researchers found that exposure to MEHP triggered apoptosis in germ cells. Therefore, we can suggest that the effects of plasticizers on testicular and germ cell apoptosis can be dose-dependent, and application period can be a major factor in the induction of testicular cell death.

Caspases are proteolytic enzymes that play essential roles in both intrinsic (caspase 9 and caspase 3) and extrinsic (caspase 8) pathways of apoptosis. There are different types of caspases with different tasks. These enzymes can be divided into 3 groups as initiators (caspase 2, 8, 9, and 10), effectors or executioners (caspase 3, 6, and 7), and inflammatory caspases (caspase 1, 4, and 5) (Gordeziani et al. 2017). In our study,

there were no statistically significant differences in testicular caspase 3 levels between the groups. However, caspase 3 levels of BPA + DEHP group were found to be 56.5% higher than that of the control but still not markedly different. This can be due to high standard deviation and low number of animals within the study groups. On the other hand, testicular caspase 8 levels in DEHP and BPA + DEHP group were found to be significantly higher than that of the control group ( $p < 0.05$ ), indicating that DEHP and combined exposures trigger the extrinsic apoptotic pathway. In a study by Borch et al. (2005), pregnant rats were orally exposed to DEHP (300, 750 mg/kg/day) or DEHP (750 mg/kg/day) in combination with di(2-ethylhexyl) adipate (DEHA, 400 mg/kg/day). Their male offspring also received the same treatments during lactation period between the 21st and 26th PND. Testicular caspase 3 levels showed no significant changes vs. control. In another study, oral single-dose (400 mg/kg body weight) of MEHP was administered to 28-day-old male Wistar rats. After 3 and 12 h, the activity of testicular caspase 3 increased significantly (Dalgaard et al. 2001). In a study performed on adult male Sprague-Dawley rats, BPA (50, 100, and 200 mg/kg) was administered orally for 4 weeks. There were increases in both caspase 3 and 9 levels in testis compared with that of control (Wang et al. 2014). Liu et al. (2013) conducted a study on ICR rats which were exposed to BPA in water (10, 100, and 1000 nmol/L) during pregnancy and lactation. Caspase 3 expressions in testes were found to be increased in male pups at 21st PND. These studies showed that the effects of BPA and DEHP exposure on caspases can be controversial, most possibly resulting from the differences between the doses, exposure periods, and animal models.

Autophagy is another type of programmed cell death. Targeted cytoplasmic constituents as well as long-lasting proteins, damaged organelles, microorganisms, and viruses are isolated from the rest of the cell within a double-membraned vesicle which is called “autophagosome”. Eventually, the autophagosome fuses with lysosomes, and later, these contents are degraded by lysosomal enzymes and recycled. Autophagic cell death occurs in four steps: triggering, vesicle nucleation, vesicle elongation, and fusion-degradation phase. It can be activated by different conditions, such as food starvation and high levels of intracellular oxidative stress (Yu et al. 2018). Numerous proteins play roles in the regulation of autophagy. Beclin 1 takes part on the vesicle nucleation phase of autophagy by forming the isolation membrane, a double-membrane structure that engulfs cytoplasmic material to form the autophagosome (Yu et al. 2018). p62 is an autophagy substrate that is used as a reporter of autophagic activity. The intracellular levels of p62 are dependent on transcriptional regulation and posttranslational autophagic degradation. Autophagy activation reduces the expression of p62. Collectively, p62 relates to the formation of ubiquitin-

positive inclusions and binds LC3II to facilitate autophagic degradation (Liu et al. 2016). LC3 is a protein having an important role in the vesicle elongation phase. During autophagy, the autophagosomes involve cytoplasmic components (cytosolic proteins and organelles). Simultaneously, the cytosolic form of LC3 (LC3-I) is combined with phosphatidylethanolamine to generate the LC3-phosphatidylethanolamine conjugate (LC3-II). LC3-II is bound to the autophagosome membrane and takes part in phagophore expansion as well as cargo recognition (Tanida et al. 2008). In our study, we observed that testicular LC3 levels of BPA group (63% increase,  $p > 0.05$ ), DEHP group (81% increase,  $p < 0.05$ ), and BPA + DEHP group (72% increase,  $p < 0.05$ ) were higher than that of the control group. In addition, Beclin 1 levels were found to be increased with BPA (10%,  $p > 0.05$ ), DEHP (61%,  $p < 0.05$ ), and BPA plus DEHP (40% increase,  $p < 0.05$ ) exposure. Testis p62 levels were decreased in BPA plus DEHP group (91%) vs. control and vs. significantly lower than that of the control group and single exposure groups ( $p < 0.05$ ). According to our results, prenatal and lactational exposures to BPA, DEHP, and BPA + DEHP can induce autophagy at the LOAEL doses of these EDCs. In a recent work, prepubertal Sprague-Dawley rats were exposed to different low doses of BPA and nanophenol separately or jointly for 4 weeks. The results indicate that the joint exposure induced excessive apoptosis and autophagy in the testes, as proved by a series of characteristics such as chromatin condensation and autophagosome formation (Su et al. 2018). Su et al. (2018) demonstrated that exposure to BPA (100, 200, 300, 400, 500, and 600  $\mu\text{M}$ ) decreased the cell viability in goat Sertoli Cells (gSCs) in a dose-dependent manner, and all results indicated that the viability of gSCs was disrupted after BPA treatment. The authors suggested that BPA induced both apoptosis and autophagy. The possible suggested mechanisms for the loss in cell viability were suggested to be high levels of intracellular ROS production and alterations in mitochondrial membrane potential (Zhang et al. 2017). In another study by Quan et al. (2017), male Sprague-Dawley rats (5 weeks) were exposed to different concentrations (0, 2, 10, and 50 mg kg/bw) of BPA for 10 days by intraperitoneal injection. The researchers found that BPA exposure led to both apoptosis and autophagy in rat testis. Male mice were exposed with different doses of DEHP (0, 100, 200, or 400 mg/kg/day) orally for 21 days in a study by Sun et al. (2018). They found that DEHP significantly induced autophagy in the testicular tissues and led to increases in LC3-II, Atg5, and Beclin-1 proteins. Another recent study by Yi et al. (2018) showed that developmental exposure to DEHP (0, 250 and 500 mg/kg/day, i.g., from postnatal day 1 to 35) simultaneously increased the number of autophagosomes and the protein expressions of autophagy

markers LC3 and p62 in rat testes, suggesting that the accumulated autophagosomes raised from impaired the degradation process in autophagy. The results of these studies support our findings. However, there is no study in literature evaluating the effects of combined exposure to BPA and DEHP on autophagy.

## Conclusion

The results of this study revealed that early-life exposure to EDCs may cause adverse effects on male reproductive system in the later stages of life. These adverse effects are suggested to be testicular histopathological changes, apoptosis, and autophagy. Although there are studies in literature that suggest the reprotoxic effects of BPA and/or DEHP, this study further contributes to literature by comparing the prenatal and postnatal cytotoxic, reprotoxic, and developmental effects of BPA + DEHP to individual exposures to BPA and DEHP. Further studies are needed to examine the combined impact of different plasticizers on the male reproductive system in order to elucidate the underlying mechanisms. As inter-species changes make it difficult to understand the effects of these chemicals on humans, more studies on different species as well as in vitro research are needed in order to understand the outcomes of combined exposures.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflicts of interest.

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