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ARTICLE

Effects of prenatal and lactational bisphenol a and/or di (2-ethylhexyl) phthalate exposure on male reproductive system

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ABSTRACT

Bisphenol A (BPA) and phthalates are abundantly used endocrine disrupting chemicals (EDCs). The aim of this study was to evaluate the effects of single and combined exposures to BPA and/or di(2-ethylhexyl) phthalate (DEHP) in prenatal and lactational period on rat male reproductive system in later stages of life. Pregnant Sprague-Dawley rats were divided randomly to four groups (n = 3/group): Control (corn oil); DEHP (30 mg/kg/day); BPA (50 mg/kg/day); and BPA+ DEHP (30 mg/kg/day DEHP and 50 mg/kg/day BPA). Groups exposed to EDCs through 6–21 gestational days and lactation period by intragastric lavage. Male offspring (n = 6/group) from each mother were fed till adulthood and were then euthanized. Later, reproductive hormones, sperm parameters, and oxidative stress parameters were determined. In conclusion, we can suggest that prenatal and lactational exposure to BPA and DEHP may cause adverse effects in male reproductive system in later stages of life especially after combined exposure.

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KEYWORDS
Bisphenol A; endocrine disruptors; phthalate; reproductive system

Introduction

Endocrine disruptors (EDCs) are synthetic or natural chemicals which mimic or inhibit the effects of hormones that disrupt the normal functioning of the body (DiVall 2013). These substances can cause reproductive and developmental problems due to their pronounced effects on endocrine system (EPA 2017). Humans are exposed to EDCs very widely and from different sources in everyday life. Synthetic EDCs can spread to the environment as a result of industrial production (Newbold 2010). In addition, the contamination of soil and groundwater can lead to direct contact of individuals in the immediate vicinity. Moreover, these chemical mixtures can accumulate in the food chain (Diamanti-Kandarakis et al. 2009; Schug et al. 2011). These substances may affect many basic processes such as growth, stress response, gender development, reproductive ability, insulin production and use, and metabolic rate by disrupting the endocrine balance (Newbold 2010).

Phthalates are phthalic acid derivatives that are used to provide flexibility, softness, and durability for plastic materials. These plasticizers are used toys, coating materials, plastic food containers, bottles, medical devices, baby products, electronic devices, and various household chemicals. Phthalates are also used as color and odor fixatives in cosmetics and personal care products. Di-(2-ethylhexyl) phthalate (DEHP) is one of the most widely used phthalate. Bisphenol A (BPA) is

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a bisphenol derivative that is used to add hardness and flexibility to polycarbonate material (Diamanti-Kandarakis et al. 2009). Because of their widespread use and easy removal from plastic material, humans are exposed to DEHP and/or BPA by oral, inhalation and dermal routes (David 2000; Pereira et al. 2015). Exposure to these substances may cause a wide variety of significant adverse effects such as abnormalities of male and female reproductive systems, obesity and metabolic disorders, neuroendocrine and behavioral disorders as well as various cancers (Diamanti-Kandarakis et al. 2009, 2010).

Today, we are usually exposed to both phthalates and bisphenol derivatives as mixtures. Combined exposures may cause additive, synergistic, or antagonistic effects (David 2000). These chemicals may alter each other's dose–response relationship and unpredictable outcomes can be observed in the entire population (Diamanti-Kandarakis et al. 2010; Pereira et al. 2015). Moreover, exposure of fetuses and infants is of particular importance as they are the most sensitive population and several papers show that the effects of EDCs may be more pronounced in early life (Heindel 2007; Wolff et al. 2008; Gluckman et al. 2008; Schug et al. 2011).

The aim of this study was to evaluate the effects of single and combined exposure to BPA and/or DEHP on rat male reproductive system in adulthood when exposure is actualized in prenatal and lactation period. These evaluations were carried out by examining sperm parameters (number, motility, and morphology), reproductive hormone levels, and oxidative stress parameters.

Materials and methods

Chemicals, reagents and kits

All chemicals, including Tris, diethylene triamine pentaacetic acid (DTPA), bovine serum albumin (BSA), phenylmethylsulfonyl uoride (PMSF), di(2-ethylhexyl) phthalate (DEHP, purity ≥99%), and bisphenol A (BPA, purity ≥99%) were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Commercial ELISA kits for rat plasma testosterone and estradiol (E2) were obtained from Cayman Chemical (Michigan, USA). Follicle-stimulating hormone (FSH), luteinizing hormone (LH), and sex hormone binding globulin (SHBG) ELISA kits were purchased from Elabscience (Houston, Texas) and dehydroepiandrosterone sulphate (DHEAS) ELISA kit was from MyBioSource (San Diego, USA). PureSperm Wash and the DiffQuick Stat III® Sperm Staining kit were from Nidacon International (Mölndal, Sweden) and MidAtlantic Diagnostics (Mt. Laurel, New Jersey, USA), respectively.

Commercial kits for detection of oxidative stress parameters [glutathione (GSH) assay kit, catalase (CAT) assay kit, superoxide dismutase (SOD) assay kit, glutathione peroxidase (GPx) assay kit, thiobarbituric acid reactive substances (TBARS) assay kit, and protein carbonyl colorimetric assay kit] were obtained from Cayman Chemical (Michigan, USA).

DNA isolation kit Quick-DNA was obtain from Zymo Research (Irvine,USA) and oxidative DNA damage kit BIOXYTECH® 8-OhdG-EIATM was purchased from Oxis International, Inc. (California, USA).

All animal feed was supplied by Gazi University Experimental Animals Laboratory (Ankara, Turkey).

Animals and dosing regimen

Di(2-ethylhexyl) phthalate was prepared in corn oil. BPA was dissolved in 1 ml of 96% ethanol and diluted with corn oil. Lowest observed adverse effect levels (LOAEL) on rat male reproductive system were chosen as applied doses for both DEHP (30 mg/kg/day) and BPA (50 mg/kg/day) (Shelby 2006; EFSA 2006; Tomza-Marciniak et al. 2017; ATSDR 2019).

All animals were supplied from Gazi University Experimental Animals Laboratory (Ankara, Turkey). Female rats of similar weight and age were randomly chosen from the animal lab. They were mated with healthy male rats. Pregnant Sprague-Dawley rats were later divided randomly to

four groups (n = 3/group): Controls received corn oil; DEHP group received 30 mg/kg/day DEHP; BPA group received 50 mg/kg/day BPA and BPA+ DEHP group received 30 mg/kg/day DEHP and 50 mg/kg/day BPA through 6–21 gestational days and lactation period by intra-gastric lavage.

Male offspring (n = 6/group) from each mother were fed until the end of twelfth postnatal week; then euthanized (cardiac exsanguination under deep anesthesia) and testes and epididymis were removed. Blood samples were drawn during euthanasia in order to obtain plasma.

Ethical approval

The animals were treated humanely and with regard for alleviation of suffering, and the study was approved by Gazi University Laboratory Animals Ethical Committee (Ethical approval number: G.U.ET-15.066).

Plasma hormone levels

Blood samples are taken to heparinized tubes during the euthanasia. The tubes are centrifuged for 10 min at 1500 × g. Plasma were separated, aliquoted, and stored at −80°C until the analysis of reproductive hormones.

Plasma FSH, LH, DHEAS and SHBG levels were measured by using spectrophotometric kits which employ sandwich-ELISA method. Testosterone and E2 levels were measured with competitive ELISA kits.

Sperm count, motility, and morphology

After removal, the right epididymis was separated into *caput* and *cauda*. Weight of the *cauda* was recorded for the calculation of sperm counts. *Cauda* was then placed into a tube containing 1 ml PureSperm Wash supplemented with 0.5% BSA, and minced with anatomic scissors. The suspension was centrifuged at 800× g for 10 min. The pellet was diluted with PureSperm Wash. About 10 µL of the suspension was applied to Neuber hemocytometer for sperm count. About 100 sperms were assessed by manual counting for sperm motility under a microscope at ×200 magnification. Sperms were categorized as 'progressive motile sperms' and 'nonmotile sperms.' The same diluted samples were also used for sperm morphology. First, 10 µL of the suspension was applied to a slide and samples were fixed after drying. These samples were later stained with the DiffQuik Stat III Sperm Staining kit. The abnormal and normal sperms were counted manually (100 sperms/slide, duplicate counting) at ×400 magnification. Sperm morphology evaluations were performed by counting normal sperms and abnormal sperms. Sperms with abnormal morphology are classified as follows: without head, without tail, with rudimentary tail, with curved tail, with coiled tail, with looped tail.

Testis tissue preparation

Left testes were removed after decapitation, washed in saline, and deionized water and were frozen immediately in liquid nitrogen. Later, testis was separated into pieces and kept at −80°C until the analysis. For oxidative stress parameters, testes tissue was homogenized with homogenization buffer (10 mM Tris/1 mM DTPA/1 mM PMSF) using a Teflon-glass homogenizer and then centrifuged at 2000 × g at 4°C for 10 min. The supernatants were further centrifuged at 20,000 × g at 4°C for 20 min and stored at −80°C until analysis.

Oxidative stress parameters and DNA damage

Testis tissue GSH levels was measured by a commercial kit, based on the reaction of the sulfhydryl group of GSH with 5,5′-dithio-bis-[2-nitrobenzoic acid (DTNB)] to produce a yellow colored

5-thio-2-nitrobenzoic acid (TNB) and in the same cycle GSH is simultaneously converted to GS-TNB. The absorbance values of the samples were measured at 414 nm. The results were expressed as nmol/mg protein.

Lipid peroxidation was assessed by a commercial TBARS assay kit which measures the concentration of malondialdehyde (MDA), a naturally occurring product of lipid peroxidation. MDA forms a complex with thiobarbituric acid (TBA) under high temperature and acidic conditions. The color intensity of MDA-TBA complex is measured at 530 nm spectrophotometrically. The calculation of the amount of MDA was made by using MDA standards and the results were expressed as nmol/mg protein.

Protein carbonyl levels, as an indicator of protein oxidation was measured by a spectrophotometric kit that is based on the reaction between 2,4-dinitrophenylhydrazine (DNPH) and protein carbonyls. The absorbance values of the samples were measured at 360 nm and the results were expressed as nmol/mg protein.

For the measurement of 8-OHdG levels, which are markers for oxidative DNA damage, DNA was isolated from the testis tissue with a DNA isolation kit which uses a genomic lysis buffer and later spin columns for extraction. Later, 8-OHdG levels were measured by a commercial kit that uses sandwich ELISA method. The absorbance of the samples or standards was measured at 450 nm. Four-parameter logistic regression curve was used. 8-OHdG levels within the samples were calculated by using Spectramax computer program and expressed as ng/ml.

Antioxidant enzymes

Testis GPx activity was measured by commercial kit which indirectly measures the GPx activity by a coupled reaction with glutathione reductase (GR). Absorbance was measured at 340 nm at one minute intervals and GPx activity was expressed as nmol/min/mg protein.

Testicular CAT activity was measured by commercial spectrophotometric kit which utilizes the peroxidative function of CAT for determination of enzyme activity. The absorbance values of the samples were measured at 540 nm and CAT activity was expressed as nmol/min/mg protein.

SOD activity was measured by a commercial colorimetric kit. The kit uses a radical detector [i.e. tetrazolium salt solution namely 2-(4-iodophenyl)−3-(4-nitrophenyl)-5-(2,4-sulfophenyl)-2 H-tetrazolium, monosodium salt, WST-1 formazan] that produces a water-soluble formazan dye upon reduction with a superoxide anion. One unit of SOD is defined as the amount of enzyme needed to exhibit %50 dismutation of the superoxide radical. The SOD activity was expressed as U/mg protein.

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 by using a Statistical Package for Social Sciences Program (SPSS) version 17.0 (SPSS Inc., Chicago, IL). p values <0.05 were considered as statistically significant.

Results

Hormone levels

Plasma hormone levels are presented in Figure 1. There were no statistically significant differences between the groups concerning LH, FSH, testosterone, and SHBG levels ($p > 0.05$; vs control and each other). BPA exposed group have lower plasma E2 levels (37%) compared to control group but no statistically significant difference was found due to high intergroup variation ($p > 0.05$). The E2 levels of BPA+DEHP group were significantly higher (2.7-fold) than control ($p < 0.05$). No

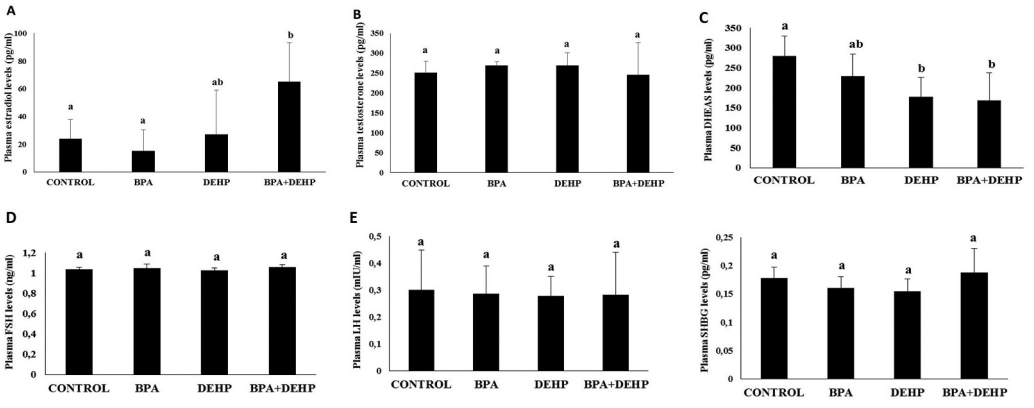

Figure 1. Plasma hormone levels in study groups. All study groups consisted of 6 animals. All results were given as mean±SD. (a) Plasma estradiol levels; (b) plasma testosterone levels; (c) plasma dehydroepiandrosterone sulphate levels; (d) plasma follicle stimulating hormone levels; (e) plasma luteinizing hormone levels; (f) plasma sex hormone binding globulin levels.BPA: bisphenol A; DEHP: di(2-ethylhexyl)phthalate E2: estradiol; DHEAS: dehydroepiandrosterone sulphate; SHBG: sex hormone binding globulin; FSH: follicle stimulating hormone; LH: luteinizing hormone [a,b]Bars that do not share same letters (superscripts) are significantly different from each other ($p < 0.05$).

significant changes were observed in the BPA group plasma DHEAS levels compared to the control group. However, plasma DHEAS levels significantly decreased in the DEHP and BPA+DEHP groups (37% and 40% respectively; $p < 0.05$ vs control).

Sperm count, motility, and morphology

The sperm counts and sperm motilities of the study groups are presented in Table 1. Sperm count was found to be significantly lower in the BPA group (55%), DEHP group (35%), and BPA+DEHP group (61%) compared to the control ($p < 0.05$). Sperm motilities of all study groups were significantly lower than the control group ($p < 0.05$, all).

In the control group, we have observed that the rats had 90.9% normal sperm and 9.1% abnormal sperm morphology. The normal sperm ratio was found to be significantly lower in all study groups vs. control and BPA+DEHP group had the lowest normal sperm ratio. Similarly, the percentage of abnormal sperm was found to be significantly higher in the BPA (39%), DEHP (28%), and BPA +DEHP (58%) groups when compared to the control. The highest abnormal sperm count was observed in the BPA+DEHP and it was markedly higher than the control and the single exposure groups ($p < 0.05$) (Table 2). The abnormal sperm morphologies in the study groups were shown in Figure 2

Oxidative stress parameters

There were no significant difference in total GSH levels in BPA and DEHP groups compared to the control group. Total glutathione levels in BPA + DEHP group were found to be significantly

Table 1. Sperm count and progressive sperm motility in the study groups.

	Control	BPA	DEHP	BPA+DEHP
Sperm count (Sperm count/g epididymis)x10^6	971.46 ± 208.96^a	433.07 ± 64.86^b	628.63 ± 61.16 ^{c}	381.75 ± 116.23^b
Progressive sperm motility (%)	71.18 ± 3.96^a	48.65 ± 5.84^b	39.49 ± 6.56^{bc}	36.62 ± 4.03 ^{c}

All results were given as mean ± SD.
All study groups consisted of six animals.
BPA: bisphenol A, DEHP: di(2-ethylhexyl)phthalate
[a]Columns that do not share same letters (superscripts) are significantly different from each other ($p < 0.05$).

Table 2. Sperm morphology motility in the study groups.

	Control	BPA	DEHP	BPA+DEHP
Normal sperm (%)	90.92 ± 3.53[a]	61.08 ± 7.25[b]	71.92 ± 9.76[b]	41.92 ± 11.10 [c]
Abnormal sperm (%)				
Headless (%)	2.33 ± 1,78[a]	5.58 ± 1.74[b]	3.75 ± 1,70[b]	9.42 ± 1,72 [c]
Tailless (%)	0.33 ± 0,52[a]	3.75 ± 2.19[b]	0.92 ± 0.97[a]	3.67 ± 2.07[b]
Rudimentary tail (%)	0.83 ± 1.43[a]	3.08 ± 2.86[b]	3.08 ± 2.44[b]	5.67 ± 2.66 [c]
Curved tail (%)	4.42 ± 1.99[a]	17.42 ± 5.7[b]	12.42 ± 5.35[b]	22.83 ± 8.48 [c]
Coiled tail (%)	0.75 ± 1.04[a]	7.83 ± 2.75[b]	7.25 ± 2.8[b]	14.42 ± 2.71 [c]
Looped tail (%)	0.42 ± 0.58[a]	1.25 ± 1.04[b]	0.67 ± 0.61[b]	2.08 ± 1.24 [c]

All results were given as mean ± SD.
All study groups consisted of 6 animals.
BPA: bisphenol A, DEHP: di(2-ethylhexyl)phthalate
[a,b,c]Columns that do not share same letters (superscripts) are significantly different from each other ($p < 0.05$).

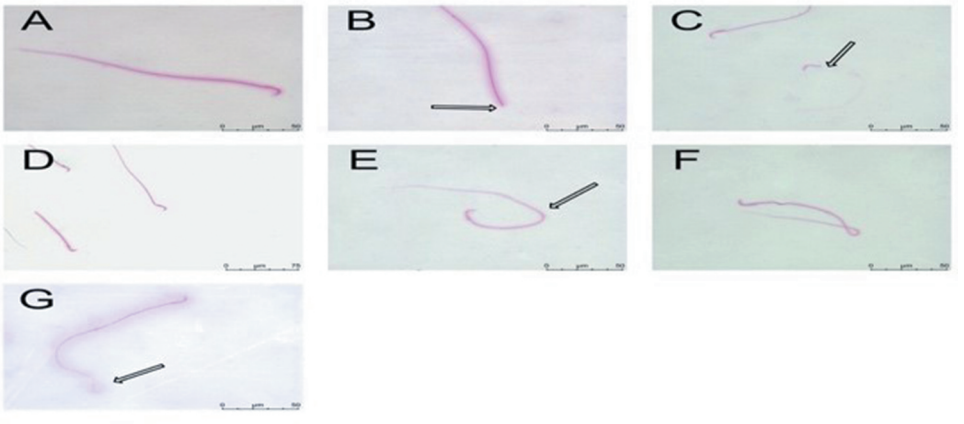

Figure 2. The abnormal sperm morphologies in the study groups. (a) Normal sperm (Control); (b) headless sperm (arrow) (BPA +DEHP); (c) tailless sperm (arrow) (BPA); (d) two normal sperm and one with rudimentary tail sperm (BPA+DEHP); (e) curved tail sperm (arrow) (BPA); (f) coiled tail sperm (BPA+DEHP); G- looped tail sperm (arrow) (BPA) DiffQuick ×400. BPA: bisphenol A, DEHP: di(2-ethylhexyl)phthalate.

decreased compared to control, BPA and DEHP groups (26%, 31%, and 27%, respectively) ($p < 0.05$) (Table 3).

Testis MDA levels were not significantly different in the BPA group compared to the control group, but these levels were found significantly higher in the DEHP and BPA+DEHP groups vs. control (28% and 60%, respectively; $p < 0.05$, both) (Table 3).

There were no significant differences in the carbonyl levels between the study groups ($p > 0.05$, all). Testicular 8-OHdG levels were also not markedly different between the study groups ($p > 0.05$, all) (Table 3).

Antioxidant enzymes

Testicular GPx activity decreased in BPA (37%), DEHP (42%), and BPA + DEHP (33%) groups compared to control group. However, this decrease was not significant since inter-group variations were high ($p > 0.05$). Testicular SOD activities of BPA (42% $p < 0.05$), DEHP (20.5% $p > 0.05$), and BPA + DEHP (20% $p < 0.05$) were lower when compared to the control group. CAT activity in testis decreased in BPA (18% $p > 0.05$), DEHP (32.5% $p < 0.05$), and BPA+DEHP (32% $p < 0.05$) groups compared to control (Table 4).

Table 3. Testis oxidative stress parameters in the study groups.

	Control	BPA	DEHP	BPA+DEHP
Total GSH (nmol/mg protein)	227.98 ± 23.64 [a]	243.73 ± 45.66 [a]	229.40 ± 35.62 [a]	168.27 ± 44.55 [b]
MDA (nmol/mg protein)	1.46 ± 0.20 [a]	1.40 ± 0.26 [a]	1.87 ± 0.29 [b]	2.35 ± 0.92 [b]
Carbonyl groups (nmol/mg protein)	0.61 ± 0.22 [a]	0.54 ± 0.09 [a]	0.65 ± 0.13 [a]	0.67 ± 0.13 [a]
8-OHdG (ng/ml)	0.77 ± 0.23 [a]	0.92 ± 0.17 [a]	0.87 ± 0.16 [a]	0.74 ± 0.09 [a]

All results were given as mean ± SD.
All study groups consisted of 6 animals.
BPA: bisphenol A, DEHP: di(2-ethylhexyl)phthalate
8-OHdG: 8-hydroxy-2′-deoxyguanosine
MDA: Malondialdehyde
GSH:Glutathion
Columns that do not share same letters (superscripts) are significantly different from each other ($p < 0.05$).

Table 4. Testis antioxidant enzyme activities in the study groups.

	Control	BPA	DEHP	BPA+DEHP
GPx (nmol/min/mg protein)	0.1262 ± 0.0459[a]	0.0796 ± 0.0289[a]	0.0724 ± 0.0143[a]	0.0945 ± 0.0309[a]
SOD (U/mg protein)	7.1039 ± 1.0656[a]	4.1630 ± 2.3785[b]	5.6445 ± 1.9455[ab]	5.6445 ± 0.8182[b]
CAT (nmol/min/mg protein)	209.3095 ± 57.4010[a]	171.3934 ± 37.6841[ab]	141.3016 ± 30.9149[b]	142.8120 ± 18.7608[b]

All results were given as mean ± SD.
All study groups consisted of 6 animals.
BPA: bisphenol A, DEHP: di(2-ethylhexyl)phthalate
GPx: Glutathione peroxidase
SOD: Superoxide dismutase
CAT: Catalase
[a]Columns that do not share same letters (superscripts) are significantly different from each other ($p < 0.05$).

Discussion

Among all the EDCs, humans are more abundantly exposed to BPA and phthalates than any other. They are used in plastic materials like toys, plastic bottles, blood transfusion bags, food containers, baby bottles, paint powders, cosmetics (perfumes, soaps, make-up products), and filling materials in dentistry. These plasticizers are suggested to have negative impact on human health and they may affect many organs, systems, and pathways. Early life exposure to these chemicals may have more pronounced consequences. Prenatal exposures are suggested to have more serious consequences in adulthood than any other exposures (David 2000; Howdeshell et al. 2008; Pereira et al. 2015; Careghini et al. 2015; Yaghjyan et al. 2016; Tsochatzis et al. 2017).

This study aimed to examine effects of prenatal exposure to BPA and/or DEHP on rat testis. The results of this study can be discussed in three parts:

Sex hormone levels

In this study, we observed that levels of FSH, LH, testosterone, and SHBG did not change in any of the study groups vs. control ($p > 0.05$, all). Plasma estradiol levels were significantly increased in the combined exposure (BPA+DEHP) group compared to the control ($p < 0.05$).

There are different studies showing that maternal BPA exposure has different effects on testicular testosterone levels. Some of the studies suggest that BPA exposure at low and high doses during prenatal and lactational periods does not affect plasma testosterone levels in male pups in mice and rats while other studies suggest marked decreases or increases (Xi et al. 2011a; Salian et al. 2009; Kobayashi et al. 2012; Gámez et al. 2014). Similarly, there are studies reporting that plasma estradiol, FSH and LH levels may be not affected, decreased or increased after prenatal BPA exposure (Borch et al. 2006; Kobayashi et al. 2012; Ma et al. 2017). In our study, prenatal BPA exposure did not have a significant effect in the sex hormone levels when compared to control. On the other hand, some studies show that

maternal DEHP exposure causes a decrease in Leydig cell testosterone production and plasma testosterone levels while others suggest no change or increment (Akingbemi et al. 2001; Andrade et al. 2006; Culty et al. 2008; Barakat et al. 2017). Akingbemi et al. (2001) reported that exposure to DEHP (100 mg/kg/day) in the gestational (GD12-21) and lactational (PND1-21) periods caused reduction in androgen levels only during the neonatal and peripubertal period. However, testosterone levels of these animals were not found to be significantly different than control after 90 days. These observations suggest that the inhibitory effect of DEHP on Leydig cells is reversible, and/or that a new and healthy Leydig cell population can develop after the phthalate exposure ends (Akingbemi et al. 2001). Moreover, different studies suggested that maternal DEHP exposure can cause decreases or increases on serum LH levels (Andrade et al. 2006; Xi et al. 2011a; Axelsson et al. 2015). A study in which, 0.1-1-10 mg/kg/day BPA and DEHP combination was administered with oral gavage to CD-1 mice at GD1-21 and PND1-21, animals showed significant decreases in plasma FSH and testosterone levels in the prepubertal period compared to control (Manikkam et al. 2013). In other studies, exposure to a combination of DEHP and BPA did not affect serum hormone (testosterone, LH and FSH) levels in rodents, in accordance with our results (Howdeshell et al. 2008; Jones et al. 2014; Isling et al. 2014; Yang et al. 2016). Moreover, nonsignificant differences in LH levels among the groups may be associated with unchanged testosterone concentrations. The discrepancy between the studies in literature may arise from different doses, different dosing intervals and different exposure periods.

DHEAS is converted to testosterone and androstendione and the decrease in DHEAS levels is thought to affect this biotransformation (Neunzig and Bernhardt 2014). In the present work, we observed that DHEAS levels were significantly decreased in the DEHP and BPA+DEHP groups vs. control. Although there was a decrease in BPA group, it was not significant. There are few studies that determine the effect of prenatal phthalate exposure on DHEAS levels. In a study evaluating the effects of prenatal phthalate exposure on boys, serum DHEAS levels were found to be decreased due to elevated some phthalate metabolite (mono-benzyl phthalate, mono-n-butyl phthalate, or mono-isobutyl phthalate) levels (Ferguson et al. 2014a). This may be interpreted as the anti-androgenic effect of exposure to phthalates, particularly to DEHP.

In a study by Culty et al. (2008), pregnant Sprague-Dawley rats were exposed to different doses of DEHP (58–1250 mg/kg/day) from GD14 to the day of parturition by oral gavage and exposure did not change plasma estradiols in male offspring (Culty et al. 2008). Another study showed that maternal DEHP (20, 200, 500, or 750 mg/kg/day) exposure from GD11 to birth led to an increase in estradiol and LH levels in adulthood while causing decreases in serum testosterone levels in males (Barakat et al. 2017). It is known that testosterone is converted to estradiol by Cyp19a1 (aromatase). Decreased testosterone levels may reduce estradiol synthesis. However, studies reporting increased serum estradiol levels in DEHP exposure have linked this increase to abnormal hepatic estrogenic metabolism. There are also studies showing that DEHP may cause liver hyperplasia and deterioration of estrogen metabolism (Eagon et al. 1994; Barakat et al. 2017). In various studies, it has been stated that DEHP may have estrogenic effects (Czernych et al. 2017; Watkins et al. 2017). In this study, the increase in estradiol levels in the BPA+DEHP group compared to the control group can be explained by the combined effects of these two EDCs on testosterone and hepatic biotransformation of estrogen.

Sperm parameters

In this study, there were significant decreases in sperm counts and marked decreases in sperm motility in all study groups compared to control. Moreover, all study groups showed increased abnormal sperm morphology vs. control. BPA+DEHP group had the highest abnormal sperm morphology and the lowest sperm motility in all of the study groups. We can suggest that prenatal exposure to EDCs, particularly combined exposures may lead to decreases in sperm count, sperm motility and sperm morphology. This may lead to an increase male infertility, as suggested by different studies in literature.

Numerous studies have shown that single and combined exposure to maternal BPA and DEHP causes a decrease in sperm count and motility (Dalsenter et al. 2006; Howdeshell et al. 2008; Culty et al. 2008; Manikkam et al. 2013; Axelsson et al. 2015; Yang et al. 2016; Ma et al. 2017). It was suggested that the effects of BPA and/or DEHP on sperm count and sperm motility is caused by their primary effect on Sertoli cells that provide the microenvironment for sperm maturation. Moreover, these effects may also arise from their direct effect on epididymis which provide sperm storage.

Wang et al. (2014) showed that adult Sprague-Dawley rats exposed BPA (50, 100, and 200 mg/kg/day, orally) had teratospermia at high doses, but not in the lowest dose (Wang et al. 2014). In a study by Aydogan et al. (2010), the researchers showed that when adult male rats were administered 25 mg/kg/day BPA orally (three times a week for 50 days), they had increased number of abnormal sperms (Aydogan et al. 2010). Li et al. (2016) observed that when Wistar male rats were orally administered 50, 100, and 200 mg/kg/day BPA in the early stages of life (aged 28 days) for 28 days, abnormal sperm count was found to be significantly increased compared to controls (Li et al. 2016).

In neonatal male CD-1 mice exposed to DEHP by hypodermic injection (0, 5, 20, and 40 μg/kg/day) from PND7 to PND48, abnormal sperm count were significantly higher compared to control (Zhang et al. 2013). Erkekoglu et al. (2011) reported that adult male rats exposed DEHP (1000 mg/kg, oral, 10 days) showed a significant increases in the number of sperms with head and tail anomalies compared to control (Erkekoglu et al. 2011). Moreover, it was suggested that prenatal DEHP exposure can lead to trans-generational effects and lead to decreased sperm production, number, and semen quality (Doyle et al. 2013). However, there are several studies that conclude that prenatal DEHP exposure does not affect sperm parameters (Xi et al. 2011b; Salian et al. 2009; LaRocca et al. 2011).

In a study performed in China, Pan et al. (2015) observed an inverse association between urinary phthalate levels of adult men and morphologically normal sperm count (Pan et al. 2015). However, two other population studies did not find a relationship between BPA and phthalate exposure and abnormal sperm morphology in adult men (Huang et al. 2014; Adoamnei et al. 2017).

Oxidative stress

In the current study, testicular antioxidant/oxidant parameters were evaluated in order to determine whether oxidative stress is one of the underlying mechanisms for the reprotoxic effects of prenatal BPA and/or DEHP exposure. Our findings show that intracellular oxidation is present, particularly in DEHP+BPA group. While no significant difference was found in testis total GSH levels in the BPA and DEHP groups, total GSH levels in the BPA+DEHP group were significantly decreased compared to the control and other groups. Testis MDA levels, an indicator of lipid peroxidation, did not change significantly in BPA group compared to control but increased significantly in DEHP and BPA+DEHP groups vs. control. Testis carbonyl levels, as indicators of protein oxidation, and testis 8-OHdG levels, as indicators of oxidative DNA base damage, were not statistically significant between the groups ($p > 0.05$). These results show that cellular membranes may get damaged and cell integrity may be impaired in particularly BPA+DEHP group. However, due to unchanged DNA base damage and protein oxidation levels, we can suggest that DNA and protein oxidation can be compensated by testicular tissue and it can be a reversible effect.

Testicular GPx activity decreased in all study groups vs. control; however, these decreases were not significant due to high standard deviation. Testicular SOD activities of BPA and BPA+DEHP groups were significantly lower than control group. SOD activity in DEHP-exposed animals was insignificantly lower than control group. CAT activity significantly decreased in the testicular tissues of all groups while the differences were significant in DEHP and BPA+DEHP groups, but not BPA group. As GPx, SOD, and CAT have important roles in the antioxidant defense system, our

results indicate that particularly in combined exposure group oxidative stress is one of the toxicity mechanisms, if not the predominant one.

Studies evaluating the effects of prenatal DEHP and/or BPA exposure on oxidative stress are limited. In a study, genistein (50 mg/kg/day) and DEHP (50 mg/kg/day, 150 mg/kg/day, 450 mg/kg/day) and the mixtures of two compounds (administered in the same doses) were administered to pre-pubertal rats by gavage (from 22th to 33th day). Testicular oxidative stress (decreased SOD, CAT and GPx activities, decreased GSH levels, increased MDA levels) was observed in DEHP treated group at 50 mg/kg/day and this condition could be mitigated by genistein (Zhang et al. 2014). Kabuto et al. (2004) were aimed to evaluate possible oxidative damage in embryonic/fetal period after BPA exposure. Female pregnant mice were exposed to BPA (5 and 10 μg/ml BPA) by drinking water during pregnancy and lactation periods and their pups were decapitated at the end of the 4th postnatal week. The results showed that high doses of BPA exposure caused increases in brain, kidney and testicular TBARS levels and decreases testicular CAT levels. Researchers suggested that exposure to BPA during embryonic and fetal periods may cause oxidative stress in different tissues of the pups ($p < 0.05$) (Kabuto et al. 2004). All these findings support our finding that prenatal exposure to DEHP or BPA may lead to oxidation in different tissues of the offspring and this phenomenon may affect sperm parameters as well as tissue integrity.

Concerning all the available data, the changes in sperm parameters, particularly in the combined exposure group may lead to decrease in fertility. The defects in sperm morphology, might affect the ability of the sperm to reach and penetrate an egg. It has been shown that round-headed spermatozoa from infertile patients contain less protamine and more histones and intermediate proteins than the normal spermatozoa (Blanchard et al. 1990). that emphasizes the fact that chromatin remolding and formation of acrosome are related processes in the late spermiogenic events. Alterations in the spermatogenic events result in the release of immature, abnormal spermatozoa in the ejaculate. Tail defects in sperms also affect sperm motility and may lead to non-progressive sperms. Proteomic research of human sperm have identified more than 1000 proteins associated with the sperm tail structures. This data highlighted the complexity of sperm tail and the possibilities of causative genes for asthenozoospermia. A large proportion of identified proteins (26%) were related to metabolism and energy production, lipid metabolism in particular. The occurrence of such proteins suggests that fatty acids are an energy substrate for sperm motility and the presence of peroxisomal pathways in sperm (Amaral et al. 2013). Identification of proteins involved in formation and transport of sperm components adds to the evidence that defects in these systems contribute to male infertility. However, having a large percentage of misshapen sperm is not uncommon. Most male fertility experts agree that the role of sperm morphology in predicting pregnancy is unclear. If 100% of the sperm are abnormal, it clearly leads to infertility. Even males have a small percent of sperms with normal morphology, mating can result in pregnancy.

Alterations in plasma sex hormone levels mainly affect the morphology and integrity of the sperm as well. Testicular cells and sperms have estrogens receptors (ERs, both ERα and ERβ) and G protein-coupled estrogen receptor 1 (GPER) and the expression of these receptors can be modified by environmental chemicals (Dostalova et al. 2017). Therefore, one of the many reasons why the sperm parameters in BPA+DEHP group are poorer vs. other groups is the significant increase in E2 levels (2.7-fold vs. control). Moreover, the decrease in plasma DHEAS levels BPA +DEHP group (40%, $p < 0.05$ vs. control) may also contribute to the significant decreases in sperm parameters in the combined exposure group as it is known that the decrease in DHEAS causes an increase in plasma DHEA levels which is associated with azoospermia (Ismael et al. 2017).

Sperm DNA is structured in a special manner that keeps the nuclear chromatin highly stable and compact. DNA damage causes promutagenic change, which in its most severe form affects the quality of the germ line and prevents fertilization (Evgeni et al. 2014). However, sperm 8-oxodG levels in all study groups were not significantly different compared to control.

We can suggest that mainly the tail defects in sperms of BPA, DEHP, and particularly in BPA +DEHP- exposed animals arise from the effects of these chemicals on sex hormone levels, lipid

metabolism, energy production and peroxisomal pathways. Literature shows that these chemicals are epigenetic carcinogens and both BPA and DEHP are known to increase peroxisome proliferation and affect fatty acid metabolism (Kassotis and Stapleton 2019). Though both of these chemicals cause some oxidative stress (as evidenced by decreases in antioxidant enzyme activities and GSH and increases in lipid peroxidation), 8-oxodG levels did not significantly increase. Therefore, it can be suggested that the main effect of the chemicals in individual or combined exposure is on peroxisomes which leads to abnormalities in sperms eventually.

In the present study, although both BPA and DEHP alone caused decreases in sperm count, progressive sperm motility and sperm abnormalities, the combined effects of these chemicals were more pronounced. It seems that combined exposure to BPA and DEHP resulted in additive reproductive toxicity. However, more mechanistic studies (i.e. proteomic and metabolomics analyses) are needed to clarify the reasons underlying our findings.

There are also population studies that link EDC exposure to oxidative stress and several pathological conditions. Some of these studies evaluated only prenatal exposure; however, the findings support our results. In one study conducted in Puerto Rico, urine and blood samples were obtained from pregnant women in the 1st, 2nd, and 3rd trimesters (n = 139) and the association between phthalate exposure, inflammation and oxidative stress was determined. Urinary oxidative stress parameters (8-OHdG, isoprostane) were found to be increased with increasing urinary phthalate levels (Ferguson et al. 2014b). In another study, urine samples were obtained from 482 pregnant women who were living in Boston between 2006 and 2008 at different weeks of pregnancy. There was a positive significant correlation between urinary phthalate levels and urinary oxidative stress parameters (8-OHdG, and 8-isoprostane) (Ferguson et al. 2015).

A cross-sectional study on healthy children (n = 41, 10–13 y) assessed the associations of urinary metabolites of bisphenols and phthalates with oxidant stress, insulin resistance, body mass, and endothelial dysfunction. Increased BPA and DEHP metabolite levels were associated with increased levels of F2-isoprostane, a marker for lipid peroxidation. Moreover, high molecular weight (HMW) phthalate metabolites was associated with significant increases in HOMA-IR units, decreased brachial artery distensibility and altered circulating levels of activated endothelial cell-derived microparticles. Bisphenol S (BPS), an analogue of BPA, was associated with marked increases albumin: creatinine ratio. The researchers concluded that exposure to bisphenols and phthalates was associated with increased oxidant stress, insulin resistance, albuminuria, as well as disturbances in vascular function in healthy children (Kataria et al. 2017). A recent study investigated the levels of BPA and 8-oxodG in 49 children with autism spectrum disorders (ASDs) (5.950 ± 1.911 y) and 40 matched controls (5.333 ± 2.279 y). The researchers found that both BPA and 8-oxodG were significantly higher in ASD group vs. control and observed positive significant correlations between both BPA and 8-oxodG with ASD severity. It was suggested that BPA may increase oxidative stress resulting in mitochondrial dysfunction that affecting the behavior and functioning of ASDs children (Metwally et al. 2018).

In conclusion, we can suggest that exposure to EDCs in the early stages of life may cause adverse effects in the male reproductive system in later stages of life. These adverse effects can be hormonal imbalance, impaired fertility due to deterioration of sperm parameters and alterations in testicular oxidant/antioxidant status. To our knowledge, this is the first study which evaluates the adverse effects of combined exposure to the most commonly used EDCs (BPA and DEHP) at both prenatal and early postnatal period on male reproductive system. Further studies are needed to clarify the underlying toxicity mechanism/s of combined exposure to EDCs. Moreover, population studies that associate combined exposure to certain pathologies are needed in order to show whether the same phenomenon in rodents is also valid for humans or not.

Disclosure statement

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