Companion animals get close to the toxic aspects of antropogenic world: cytotoxicity of phthalates and bisphenol A on dog testicular primary cells

Koray Tekin · Pinar Arslan · Beste Cil · Ayhan Filazi · Ergun Akçay · Begum Yurdakok-Dikmen

Received: 4 December 2019 / Accepted: 11 May 2020 / Published online: 20 May 2020
© Springer Nature B.V. 2020

Abstract Phthalates, which are among the most abundant plasticizers, have detrimental effects on the reproductive system. Similar to human, dogs are prominently exposed to phthalates in daily routines at low concentrations; while toys, training devices and commercial dog foods are considered as the primary sources of exposure. This study aimed to reveal and compare the cytotoxic effects of selected phthalates (Benzyl butyl phthalate (BBP), Diethyl phthalate (DEP), Bis (2-ethylhexyl) phthalate (DEHP), Di-‘isobutyl’ phthalate (DIBP), Di-‘isodecyl’ phthalate (-DIDP) Di-‘isononyl’ phthalate (DINP), Dimethyl phthalate (DMP), Di-*n*-octyl phthalate (DNOP)), and Bisphenol A (BPA) following 24 h exposure on primary testicular parenchymal cells of dog in vitro at concentrations between 0.001 and 2.5 nM. According to cytotoxicity results, DEHP was found to be the

most toxic phthalate with IC₅₀ at 22.53 μM; while DMP was the least (169.17 nM). IC₅₀ of BPA was 161.81 nM, less than the average (61.95 nM) of phthalates. In addition, dog primary testicular cells were found more susceptible to the high molecular weight phthalates (DNOP, DEHP, DINP, DIDP) than low molecular weight phthalates (DMP, DEP, DIBP, BBP). Further studies should focus on morphological, physiological and molecular differences to comprehend the mechanisms involved as well as decreasing the risk for impaired spermatogenesis caused by environmental toxicants in companion animal medicine.

Keywords Bisphenol A · Phthalates · Canine testicular cells · In vitro toxicity

K. Tekin · B. Cil · E. Akçay
Department of Reproduction and Artificial Insemination,
Faculty of Veterinary Medicine, Ankara University,
Diskapi, 06110 Ankara, Turkey

P. Arslan
Department of Biology, Faculty of Science, Ankara
University, Tandogan, 06100 Ankara, Turkey

A. Filazi · B. Yurdakok-Dikmen (✉)
Department of Pharmacology and Toxicology, Faculty of
Veterinary Medicine, Ankara University, Diskapi,
06110 Ankara, Turkey
e-mail: yurdakok@veterinary.ankara.edu.tr;
byurdakok@yahoo.com

Highlight

- Human and dog share similar environment where pollutants such as plasticizers lead reproductive disorders in both species.
- DEHP was the most toxic on primary testicular cell culture of dog.
- Comparative evaluation of endocrine disrupters on male reproductive system would allow us to understand the protective mechanisms involved.

Introduction

Phthalates and Bisphenol A (BPA) are the most common synthetic chemicals, widely used in automotive, personal care consumer product and medical industries. Phthalates, also called plasticizers, are a group of chemical substances used to soften plastics. Some phthalates have shown detrimental effects on the endocrine and reproductive system. Recently, the following 6 phthalates, Dibutyl phthalate (DBP), Bis (2-ethylhexyl) phthalate (DEHP), Benzyl butyl phthalate (BBP), Di-‘isononyl’ phthalate (DINP), Di-‘isodecyl’ phthalate (DIDP) and Di-*n*-octyl phthalate (DNOP) have been accepted as restricted substances by the European Union (EU), Japan, as well as by US Consumer Products Safety Improvement Act (CPSIA) and restricted in plasticized material in toys and childcare articles. DEHP, DBP, BBP and DIBP were listed as the restricted substances by Directive (EU) 2015/863 (2015) of the European Union, while in 2017 they were listed as endocrine-disrupting chemicals to be included into the list of REACH substances of very high concern and they are soon to be restricted for all electrical and electronic equipment as well by the EU-ANNEX XVIII (Consumer Product Safety Commission 2017).

Bisphenol A is a chemical substance widely used to make epoxy resins and polycarbonate plastics. Currently it is classified as reproductive toxicant category 1B, which provides clear evidence of an adverse effect on sexual function and fertility or on development, largely based on data from animal studies (2015). BPA is banned in baby bottles in both USA and EU as well as China and the use of it is limited for food contact materials or articles (ChemsafetyPro 2019). However, canine toys and training devices still might be an important source for the exposure of dogs to phthalates and Bisphenol A (Wooten and Smith 2013). Since the dogs are the lifetime toddlers, the possibility to chew and swallow these toys is a very high concern and it can result in long-time exposure to their gastrointestinal system. Besides, 12 chemicals (DEHP, 7 polychlorinated biphenyls, 4 polybrominated diphenyl ether congeners) have been detected in 15 commercial dog foods and they were detected in dog testes as well (Lea et al. 2016).

High molecular weight phthalates (HMWP) including BBP, DEHP, and DNOP are mostly utilized as plasticizers in polyvinyl chloride materials. In recent

years, the use of DINP and DIDP are replaced with DEHP in these areas. Low molecular weight phthalates (LMWP) including Diethyl phthalate (DEP), Diisobutyl Phthalate (DIBP), and Dimethyl phthalate (DMP) are mainly found in cosmetics and personal care products (Wu et al. 2017). It has been suggested that LMWP cause more potent toxicity even with lower concentrations (The Danish Environmental Protection Agency 2013).

Phthalates are able to cross the placenta and can induce teratogenic effects (Saillenfait et al. 1998; Fennell et al. 2004), transported to breast milk (Dostal et al. 1987; PARMAR et al. 1988), and are also found in amniotic fluid (Silva et al. 2004). Besides, they are measured in urine at whole life stages (Sathyanarayana et al. 2008). General effects of the phthalates and Bisphenol A on male reproductive health are congenital abnormalities (cryptorchidism and hypospadias), testicular abnormalities affecting its function, hypotestosteronemia, increased frequency of abnormal spermatozoa rate and higher DNA damage in germ cells as well as declined sperm count (Skakkebaek et al. 2001). However, these adverse effects vary between the species (Habert et al. 2014). Most of these exposure tests are being conducted in rodents, especially in rats, however the variation of the adverse effects on different species makes it challenging to generalize these effects and to reveal the reprotoxicity of the phthalates, where some of these adverse effects in different animal species at cellular levels such as sperm and embryonal effects might have been overlooked (Skakkebaek et al. 2016).

Recently, in vitro systems are replacing the in vivo models in order to obtain a clear insight of chemical-induced toxicity in animals, enable rapid monitoring of the toxicologic assays as well as analyze the estrogenic, androgenic and progestogenic activity in complex environmental samples (Filazi and Yurdakök-Dikmen 2018). Cell culture systems are mainly used in vitro models to determine the toxicity at the cellular level and due to their reliability, reproducibility and affordability as well as their convenience regarding the animal welfare and ethics (National Research Council (US) Committee on the Health Risks of Phthalates 2008). Species-specific reproductive toxicity battery systems as an alternative approach are expected to be developed soon, as currently available tests are extrapolated to human only and not for companion animals. As an initial step to be

further developed, primary dog testicular parenchymal cells were tested for commonly encountered environmental contaminants in vitro to develop a sensitive, reproducible male reprotox model for dogs and to evaluate the effects of selected phthalates and Bisphenol A.

Materials and methods

Animals

Parenchymal cell cultures were derived from right and left testis of three healthy dogs after the routine surgical castration following the demand of their owners. Among the dogs, two of them were hybrids that were 8 months and 2 years old. The other dog was a 1 year old pug. The testis was obtained and kept in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Chemical) containing penicillin–streptomycin solution (Sigma Chemical) under the sterile conditions. All the experiments were conducted considering the animal welfare and the ethical principles were followed. The study was approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, Ankara University, Turkey.

Primary dog testis parenchymal cell culture

The primary testis parenchymal cell cultures were made according to Yurdakok-Dikmen et al. 2017 and under the laminar flow Class 2 (BioSafety Cabinet Telstar Bio II Advance 4, Terrassa, Spain). The cell cultures chemicals were obtained from Sigma Chemical. The testicular tissues were washed three times with phosphate-buffered saline (PBS) containing penicillin–streptomycin and amphotericin B. Then, the tissues were dissected with sterile scissors and pliers. The parenchyma tissue was identified, separately minced into 3 mm pieces and incubated for 4 h at 5% CO₂ 37 °C incubator in 4 mL 0.125% Trypsin solution including 0.5 mL penicillin–streptomycin and 0.25 mL amphotericin B. Afterward, 2 mL of DMEM (containing 20% fetal bovine serum) was added to Petri dishes and incubated for 24 h. After an incubation period, the cells were filtered using 100 µm cell strainer (Falcon, Franklin Lakes, NJ, USA) and transferred into 25 mL flasks. Morphology and proliferation of the cells were examined by using live-cell

analyzer (Juli FL, Seoul, Korea) using Juli FL software.

Chemicals

Each standards of phthalates (10 µg/mL in 99% purity acetonitrile) (DMP, DEP, DiBP, BBP, DNOP, DEHP, DINP and DIDP) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). BPA(> 99%) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). DMSO was used to dilute the main stock (2500 µg/mL) while further dilutions were made using DMEM. Except DINP all were in liquid form. The main stocks of 2500 µg/mL were referred as 12.87 mM, 11.25 mM, 8.98 mM, 8 mM, 6.40 mM, 6.40 mM, 0.24 mM, 5.60 mM and 10.95 mM for DMP, DEP, DiBP, BBP, DNOP, DEHP, DINP, DIDP and BPA, respectively in Molar concentrations.

Cytotoxicity assays

Cells were plated at a density around $2 \pm 0.39 \times 10^6$ per mL (calculated by Juli Br software) to be transferred as 100 µL volume to 96 well microplates for cytotoxicity assays. Stock solution is half diluted using DMEM into 12 serials. After preincubation at 5% CO₂ 37 °C incubator for 24 h, 25 µL of test solution was then added to media. Each plate had two control groups; negative control (% 100 viable, where medium as a vehicle is included) and positive control (%0 viable, where cells are lysed using $1 \times$ Triton X). Testicular cells from three different animals were cultured in four plates. Each concentration is applied at triplicates for each plate.

After 24 h incubation, standardized cell viability test, MTT, was done according to our previous studies (Yurdakok et al. 2015). Viability of the cells was analyzed by using a microplate reader (SpectraMax i3/i3x MultiMode Detection Platform, Molecular Devices, Sunnyvale, CA) at 540 nm. For this purpose, the viability of cells were evaluated by $[1 - (S_{OD} - NC_{OD}) / (PC_{OD} - NC_{OD})] \times 100$ equation. In this equation, S_{OD} means sample optic density, PC_{OD} means positive control optic density, NC_{OD} means negative control optic density.

Statistical analysis

Percent inhibition, from viability values are calculated from 100% viable and 0% viable control groups as described above using Microsoft Excel program. The calculated values are then transferred to GraphPad Prism (version 5.0, GraphPad Software Inc., San-Diego, CA) program for IC₅₀ (half-maximal inhibitory concentration) calculation according to best fit regression curve. The statistical differences between the tested phthalates and BPA were analyzed following parametric test assumption using one-way analysis of variance (ANOVA) followed by Tukey's test; the differences of low and high molecular weight compounds are analyzed using Two Independent Samples Unequal Variance (Welch's Test) where significance level of $p = 0.05$ is accepted. SPSS software (Version 17, SPSS Inc., Chicago, IL) was used for statistical analyses.

Results

Morphology of primary dog testis parenchymal cell culture

In the present study, *in vitro* toxicity of phthalate exposure was investigated on the primary testicular parenchyma cells of dogs. The parenchyma of the testis are consist of two main compartments that are the seminiferous tubules including the Sertoli cells, germ cells and sperm cells in various stages of maturation and the intertubular part, which consists of Leydig cells, fibers blood and lymphatic vessels and cells of the connective tissue (Lara et al. 2018). Parenchymal cells of three healthy dogs were cultured to evaluate the reprotoxic effects of the subjected phthalates and BPA.

Cultured cells were examined morphologically; where the parenchymal cells were round with $8.34 \pm 1.09 \mu\text{m}$ in average diameter. Cells were confluent approximately 2–3 days following culture. Primary parenchyma cells are shown in Fig. 1 following 5 days of incubation.

Following phthalate application, structural changes including disruption in membrane integrity and apoptotic bodies were visible. At higher concentrations, detachment in culture with degenerated cells were observed (Fig. 2). No significant difference between

Fig. 1 Primary dog testis parenchyma cells in 5 days. **a** First day after trypsin incubation. **b** Second day after 24 h incubation. **c** Third day after filtered and put on 25 mL flask. **d** Fourth day in 25 mL flask. **e** Fifth day in 25 mL flask (red arrow, the parenchyma cells). (Color figure online)

Fig. 2 DEHP treated cells at 16.02 nM. Arrows indicate cellular debris and necrotic cells with altered cellular morphology

the tested phthalates were observed in terms of morphological changes; while DEHP were found to initiate the structural changes at low concentrations (0.625 nM). Parent compound DEP were more toxic than its metabolite DMP.

Cytotoxicity assays

According to the results of MTT cytotoxic assay, the most toxic compound was DEHP (22.53 ± 1.26 nM,

8.80 ng/mL) while the least toxic compound was the metabolite DMP (169.17 ± 22.42 nM, 32.85 ng/mL) (Table 1). In addition, phthalates were found more toxic to the parenchymal cells of dog testis, with an average IC_{50} of 61.9 ± 7.32 nM, on the contrary to Bisphenol A, with the IC_{50} value of 161.81 ± 33.76 nM, 36.94 ng/mL. The toxicity of high molecular weight phthalates was 39.57% higher on average (IC_{50} average 53.92 nM) than the average of low molecular metabolites (IC_{50} average 75.30 nM); while the results were statistically insignificant ($p > 0.05$). Recorded images following the exposure to the selected phthalates and BPA at 0.1 nM concentrations are presented at Fig. 3.

Discussion

Previous studies have revealed that phthalates can both engage to androgen binding proteins (ABP) and androgen receptors leading to an increase of ABP in the Sertoli cells which might lead to cellular death and cytotoxic effects in cells; where higher molecular weight compounds might accumulate in the cell membrane leading disruption (Wan et al. 2013). According to Wang et al. (Wang et al. 2013), DNP, DEHP, and BBP significantly reduced the expression of androgen receptors and favored apoptosis in induced pluripotent stem cells from bovine testicular cells.

Although many of the subjected phthalates have been studied in laboratory animals regarding their effects on the pregnancy and the abnormalities of the

Table 1 IC_{50} values (nM) of tested phthalates and Bisphenol A on primary cells of dog testicular parenchyma in vitro

Tested chemicals	Molecular weight (g/mol)	IC_{50} (nM) ($\pm$ SD)
DMP	194.18	169.17 ± 22.42
DEP	222.24	30.69 ± 9.39
DIBP	278.35	26.05 ± 2.86
BBP	312.37	35.06 ± 3.56
DNOP	390.56	50.67 ± 7.07
DEHP	390.56	22.53 ± 1.26
DINP	418.61	57.69 ± 7.97
DIDP	446.67	103.79 ± 4.07
BPA	228.29	161.81 ± 33.76

DMP Dimethyl phthalate, DEP Diethyl phthalate, DiBP Di-‘isobutyl’ phthalate, BBP Benzyl butyl phthalate, DNOP Di-*n*-octyl phthalate, DEHP Bis (2-ethylhexyl) phthalate, DINP Di-‘isononyl’ phthalate, DiDP Di-‘isodecyl’ phthalate, BPA Bisphenol A

Fig. 3 Control and tested compounds at subtoxic concentrations (0.14 nM DiDP, Di-‘isodecyl’ phthalate; 0.09 nM DiBP, Di-‘isobutyl’ phthalate; 0.06 nM DNOP, Di-*n*-octyl phthalate; 0.12 nM DMP, Dimethyl phthalate; 0.10 nM BBP, Benzyl butyl phthalate; 0.06 nM DEHP, Bis (2-ethylhexyl) phthalate; 0.14 nM DEP, Diethyl phthalate; 0.13 nM DINP, Di-‘isononyl’ phthalate; 0.07 nM BPA, Bisphenol A

offspring (developmental toxicity) there are very few studies that considered their toxicity on male fertility, and even less in *in vitro* materials. Most common effects of these phthalates on males, caused either with direct oral exposure or through the teratogenic effects during the pregnancy are changes in the testosterone concentrations, congenital reproductive abnormalities (cryptorchidism and hypospadias), functional abnormalities of the testicles, decreased semen quality

including increased sperm abnormalities, reduced sperm production and DNA damage in germ cells (Skakkebaek et al. 2001).

Testicular dysgenesis syndrome (TDS) is defined as a complex of disorders that includes the hypospadias and cryptorchidism in the early neonatal phase and subfertility and testicular cancers originate after the puberty. Various factors involved in TDS such as the abnormal function of Sertoli, Leydig or germ cells (Skakkebaek et al. 2001). Atrophic, Sertoli cell only, and, carcinoma *in situ* lesions which are accepted as the precursors to seminoma neoplasm, were identified as the similar changes in dogs with human TDS (Grieco et al. 2008a, b). Androgen deficiency during the fetal life that is leading to testicular cancers, malformations of the reproductive tract and impairment in the production of germ cells, is hypothesized to be the origin of this syndrome (Sharpe et al. 2011). Thus, the focus is directed towards the exposure to phthalates during the pregnancy and recently, *in utero* exposure of rats to phthalates has strengthened the hypothesis since it caused delayed maturation of the gonocyte, hypospadias, and cryptorchidism, a TDS-like phenotype (Fisher et al. 2003). Besides, a specific timeline has been identified for the endocrine-disrupting chemicals to adversely affect the reproductive tract irreversibly, which is during the programming of masculinization and it corresponds to 15–17 days of fetal period in rats and around 8–14 weeks of gestation in humans (Welsh et al. 2008; MacLeod et al. 2010).

DEHP is one of the most common phthalates and has been studied intensively both in humans and other species (Rozati et al. 2002; Oskam et al. 2005; Pant et al. 2010; Pocar et al. 2012). Besides, in dry and wet dog food, 0.20 ± 0.03 and 0.37 ± 0.10 $\mu\text{g/g}$ concentrations were detected respectively (Lea et al. 2016), while mean DEHP values were detected as 0.182 ± 0.044 $\mu\text{g/g}$ in 25 canine testis explants and have been linked with decreased linearity, straightness and velocity of sperm *in vitro* (Lea et al. 2016). Environmental concentrations of DEHP and DBP have been linked with decreased sperm motility both *in vivo* and *in vitro* in humans as well (Pant et al. 2010).

In another study that evaluated the reproductive toxicity in an andrological point of view, rats were exposed orally to 20,000 ppm DMP for 10 days and subsequently, testosterone concentrations were reduced significantly (Bingham et al. 2001). However,

while in some cases feeding the male rats with phthalate esters resulted in a decreased level of testosterone as in for DMP and DEP, some phthalates such as DBP, DiBP and DEHP had increased this level (Oishi and Hiraga 1980). Besides, several structural changes such as focal dilation and vesiculation of smooth endoplasmic reticulum, mitochondrial swelling and increased activity of interstitial macrophages were determined in the Leydig cells of the rats fed daily with 2000 mg/kg body weight DEP, DEHP or DNOP for 2 days (Jones et al. 1993).

In the present study, DMP was determined as the least toxic phthalate with an IC_{50} value of 169.1 nM, whereas DEHP has shown the most toxic effects, with an IC_{50} value of 22.5 nM, on the primary cells of dog testicular parenchyma. In our previous study (Yurdakok-Dikmen et al. 2019), the effects of seven phthalates (BBP, DBP, DIBP, DINP, DEHP, DEP and DMP) were tested on bovine primary testicular and epididymal cells in vitro. According to the obtained results, IC_{50} values were ranged from 0.15 to 4.11 ng/mL for the parenchymal cells whereas a range of 0.01–7.31 ng/mL was observed for mediastinum wherein the cytotoxic effects were more evident compared to parenchymal section. Even though each phthalate has its unique level of toxicity pattern regarding the selected tissues (caput, corpus, cauda parts of the epididymis and mediastinum and parenchymal part of the testicles), the most vulnerable tissue was the corpus epididymis followed by the mediastinum while the caput part of the epididymis well-tolerated the toxicity of the higher concentrations. Although the gradation of toxicity for the selected phthalates was similar to the bovine parenchymal cells, it was clear that bovine testicular cells were more susceptible to the toxicity of phthalates regarding the IC_{50} values.

In another in vitro research, human sperm cells were incubated with DMP, and a decline in sperm viability (%) and linearity of sperm movement (%) was observed starting from the concentrations of 0.4 mM and 0.6 mM respectively. In addition, the authors have found that 1 mM concentration of DEHP significantly decreased by 25% of the motile sperm number as well (Fredricsson et al. 1993). Moreover, elevated levels of urinary phthalates in humans have been linked with diminished sperm count and increased apoptosis and DNA damage (Wang et al. 2015). Furthermore, both their urinary metabolites

(Axelsson et al. 2015) and esters in human semen correlated negatively with the motility of sperm cells (Pant et al. 2010). Although DEHP has been shown to exhibit pro-androgenic activity, its primary metabolite mono-2-Ethylhexyl phthalate (MEHP) has an anti-androgenic activity (Chauvigné et al. 2009). A tendency of negative correlation has been found between milk concentration of MEHP and free testosterone levels of 3 months old boys ($r = -0.169$, $P = 0.107$) while a positive correlation has been observed with inhibin B ($r = 0.185$, $P = 0.075$) (Main et al. 2006). In a different study, conducted on testicular cell culture of Shiba goats in vitro, necrotic Sertoli cells with ruptured plasma membrane, vacuolization in the cytoplasm, swollen nucleus and mitochondria have been detected due to MEHP incubation with the concentration of $1 \text{ nmol} \times \text{mL}^{-1}$ for 1 h, although, in spermatogenic cells, the rupture of the nuclear membrane was evident after 6 h of incubation (Andriana et al. 2004). It has been reported that the Sertoli cells are the primary target cells in the testis (Boekelheide et al. 2005), and the detachment of spermatogenic cells are caused by the alterations on Sertoli cell (Li et al. 1998). Heindel and Chapin, (Heindel and Chapin 1989) have proposed that the toxicity of phthalate esters may relate to the Sertoli cell cAMP second messenger system since it has been concluded that the cAMP is sensitive to phthalate esters. Besides, phthalate esters inhibit the FSH-stimulated elevation of extracellular cAMP in cultured Sertoli cells (Foster et al. 2000). Despite the adverse effects of DEHP on sperm quality, it has been concluded that relatively high levels or a metabolic breakdown in culture is needed in order to observe its androgenic activity (Chauvigné et al. 2009; Lea et al. 2016).

According to our previous study in bulls (Yurdakok-Dikmen et al. 2019), the toxicity of the HMWP in average for the tested primary cultures (IC_{50} average 1.79 ng/mL) was higher than the LMWP (IC_{50} average 3.64 ng/mL); similar to the spermiotoxicity results (1.71 and 2.12 ng/mL, respectively). In accordance with the previous research in the bulls, our results were contradicted with the general hypothesis that the LMWP have adverse effects at lower concentrations (The Danish Environmental Protection Agency 2013). In the present study, the toxicity of HMWP was found 39.57% higher on average (IC_{50} average 53.95 nM) than the average of LMWP (IC_{50}

average 75.30 nM). This disagreement may be due to the different mechanism of the compound- and species-specific toxicity effects of the phthalates as well as the toxicity toleration of the affected tissue, as mentioned previously.

BPA is classified as xenoestrogens, an endocrine-disrupting chemical (EDC) with estrogenic properties and accordingly, may contribute to the development of hormone-dependent cancers (Helmestam et al. 2014). Endogenous 17 β -estradiol (E2) in vitro and in vivo can be mimicked through the binding of the BPA to the estrogen receptor and modulating target gene expression (Singleton et al. 2004). In rodents, following the exposure to BPA, reduced serum testosterone levels were observed (Ye et al. 2011). In the present study, BPA has found less toxic to parenchymal cells of dog testis with IC₅₀ of 161.81 μ M contrary to the phthalates with an average IC₅₀ of 61.95 μ M. In parallel with our results, apoptosis-induced cell death was observed in Sertoli cell cultures of rat, exposed to 150–200 μ M BPA, while BPA at < 100 μ M had slight cytotoxic effects (Iida et al. 2003). Corroborating the hypothesis, increased concentration of BPA in seminal plasma has been linked to infertility in men (La Rocca et al. 2015; Vitku et al. 2016). In addition, there is evidence that BPA adversely affects the motility, mitochondrial activity, and viability of bovine spermatozoa in vitro and by the increased intracellular formation of superoxide radicals; it can cause a mitochondrial dysfunction starting from the doses higher than 100 mg/mL (Lukacova et al. 2015).

As the morphological structures differ, the toxicity responses and underlying molecular mechanisms are expected to differ in species-specific basis. Furthermore, the combined effects of phthalates can alter the semen quality more than the effect of a single substance, depending on the concentration ratio (Lea et al. 2016). Compared to our previous study (Yurdakok-Dikmen et al. 2019) regarding the effects of phthalates on bovine testicular parenchymal cells; dog cells were found less susceptible. Therefore, even though, various researchers agree on the opinion that testicular cell cultures are valuable tool regarding the clarification of the mechanism in testicular damage and screening compounds (Gray and Beamand 1984); these species-specific differences should be considered as a potential tool for comparative male reproductive toxicity.

Conclusions

The focus on the risks of phthalates on reproduction relies mainly on human medicine; while food-producing and companion animals are often disregarded. As the phthalate contamination is a global and ubiquitous public health problem where exposure through food, water, air, and skin absorption on a continuous basis is everywhere; focus on farm and companion animals should be considered as well. The anti-androgenic phthalates (DEHP, DINP, DBP, DIBP, BBP, DPP, and DNHP) should further be considered regarding their combined effects as well as cumulative effect along with the species-specific basis for a complete risk assessment.

Acknowledgement We thank Ankara University Faculty of Veterinary Medicine for providing the infrastructure and chemicals for the analysis. All datasets used in this study are available upon request from the corresponding author.

Author contributions All the listed authors have made substantial contributions to the research design, analysis and interpretation of data and to drafting the paper and revising it critically.

Compliance with ethical standards

Conflict of interest None of the authors has any conflict of interest to declare. There has been no financial support for this work that could have influenced its outcome.

References

- Andriana BB, Tay TW, Maki I et al (2004) An ultrastructural study on cytotoxic effects of mono(2-ethylhexyl) phthalate (MEHP) on testes in Shiba goat in vitro. *J Vet Sci* 5:235. <https://doi.org/10.4142/jvs.2004.5.3.235>
- Axelsson J, Rylander L, Rignell-Hydbom A et al (2015) Phthalate exposure and reproductive parameters in young men from the general Swedish population. *Environ Int* 85:54–60. <https://doi.org/10.1016/j.envint.2015.07.005>
- Bingham E, Cohns B, Powell CH, John Wiley & Sons I (2001) Patty's toxicology, 5th edition in 9 volumes. Chem Heal Saf 8:45–46. [https://doi.org/10.1016/s1074-9098\(01\)00241-6](https://doi.org/10.1016/s1074-9098(01)00241-6)
- Boekelheide K, Johnson KJ, Richburg JH (2005) Sertoli cell toxicants. In: Skinner MK, Girsworld MD (eds) Sertoli cell biology. Elsevier Science, New York, pp 345–382
- Chauvigné F, Menuet A, Lesné L et al (2009) Time- and dose-related effects of di-(2-ethylhexyl) phthalate and its main metabolites on the function of the rat fetal testis in vitro. *Environ Health Perspect* 117:515–521. <https://doi.org/10.1289/ehp.11870>

- ChemsafetyPro (2019) Bisphenol A (BPA). In: ChemsafetyPro. [https://www.chemsafetypro.com/Topics/Restriction/Bisphenol_A_\(BPA\).html](https://www.chemsafetypro.com/Topics/Restriction/Bisphenol_A_(BPA).html). Accessed 2 Dec 2019
- Consumer Product Safety Commission (2017) Prohibition of Children's Toys and Child Care Articles Containing Specified Phthalates. Final rule. Fed Regist 82:49938–49982
- Directive (EU) 2015/863 (2015) Commission Delegated Directive (EU) 2015/863—of 31 March 2015—amending Annex II to Directive 2011/65/EU of the European Parliament and of the Council as regards the list of restricted substances
- Dostal LA, Weaver RP, Schwetz BA (1987) Transfer of di(2-ethylhexyl) phthalate through rat milk and effects on milk composition and the mammary gland. *Toxicol Appl Pharmacol* 91:315–325. [https://doi.org/10.1016/0041-008x\(87\)90054-8](https://doi.org/10.1016/0041-008x(87)90054-8)
- Fennell TR, Krol WL, Sumner SCJ, Snyder RW (2004) Pharmacokinetics of dibutylphthalate in pregnant rats. *Toxicol Sci* 82:407–418. <https://doi.org/10.1093/toxsci/kfh294>
- Filazi A, Yurdakök-Dikmen B (2018) Endokrin Bozucu Kimyasal Maddelerin Belirlenmesinde Kullanılan İn Vitro Yöntemler. *Türkiye Klin Vet Sci Toxicol Top* 4:15–21
- Fisher JS, Macpherson S, Marchetti N, Sharpe RM (2003) Human “testicular dysgenesis syndrome”: a possible model using in utero exposure of the rat to dibutyl phthalate. *Hum Reprod* 18:1383–1394. <https://doi.org/10.1093/humrep/deg273>
- Foster PMD, Cattley RC, Mylchreest E (2000) Effects of di-*n*-butyl phthalate (DBP) on male reproductive development in the rat: implications for human risk assessment. *Food Chem Toxicol* 38:S97–S99. [https://doi.org/10.1016/s0278-6915\(99\)00128-3](https://doi.org/10.1016/s0278-6915(99)00128-3)
- Fredricsson B, Möller L, Pousette Å, Westerholm R (1993) Human sperm motility is affected by plasticizers and diesel particle extracts. *Pharmacol Toxicol* 72:128–133. <https://doi.org/10.1111/j.1600-0773.1993.tb00303.x>
- Gray TJB, Beamand JA (1984) Effect of some phthalate esters and other testicular toxins on primary cultures of testicular cells. *Food Chem Toxicol* 22:123–131. [https://doi.org/10.1016/0278-6915\(84\)90092-9](https://doi.org/10.1016/0278-6915(84)90092-9)
- Grieco V, Riccardi E, Greppi GF et al (2008a) Canine testicular tumours: a study on 232 dogs. *J Comp Pathol* 138:86–89. <https://doi.org/10.1016/j.jcpa.2007.11.002>
- Grieco V, Riccardi E, Veronesi MC et al (2008b) Evidence of testicular dysgenesis syndrome in the dog. *Theriogenology* 70:53–60. <https://doi.org/10.1016/j.theriogenology.2008.02.009>
- Habert R, Muczynski V, Grisin T et al (2014) Concerns about the widespread use of rodent models for human risk assessments of endocrine disruptors. *Reproduction* 147:R119–R129. <https://doi.org/10.1530/rep-13-0497>
- Heindel RJ, Chapin RE (1989) Inhibition of FSH-stimulated cAMP accumulation by mono(2-ethylhexyl) phthalate in primary rat sertoli cell cultures. *Toxicol Appl Pharmacol* 97:377–385. [https://doi.org/10.1016/0041-008x\(89\)90342-6](https://doi.org/10.1016/0041-008x(89)90342-6)
- Helgestam M, Davey E, Stavreus-Evers A, Olovsson M (2014) Bisphenol A affects human endometrial endothelial cell angiogenic activity in vitro. *Reprod Toxicol* 46:69–76. <https://doi.org/10.1016/j.reprotox.2014.03.002>
- Iida H, Maehara K, Doiguchi M et al (2003) Bisphenol A-induced apoptosis of cultured rat Sertoli cells. *Reprod Toxicol* 17:457–464. [https://doi.org/10.1016/s0890-6238\(03\)00034-0](https://doi.org/10.1016/s0890-6238(03)00034-0)
- Jones HB, Garside DA, Liu R, Roberts JC (1993) The influence of phthalate esters on leydig cell structure and function in vitro and in vivo. *Exp Mol Pathol* 58:179–193. <https://doi.org/10.1006/exmp.1993.1016>
- La Rocca C, Tait S, Guerranti C et al (2015) Exposure to endocrine disruptors and nuclear receptors gene expression in infertile and fertile men from Italian areas with different environmental features. *Int J Environ Res Public Health* 12:12426–12445. <https://doi.org/10.3390/ijerph121012426>
- Lara NLM, Costa GMJ, Avelar GF, et al (2018) Testis physiology—overview and histology. *Encycl Reprod* 105–116
- Lea RG, Byers AS, Sumner RN et al (2016) Environmental chemicals impact dog semen quality in vitro and may be associated with a temporal decline in sperm motility and increased cryptorchidism. *Sci Rep*. <https://doi.org/10.1038/srep31281>
- Li L-H, Jester WF, Orth JM (1998) Effects of relatively low levels of mono-(2-ethylhexyl) phthalate on cocultured sertoli cells and gonocytes from neonatal rats. *Toxicol Appl Pharmacol* 153:258–265. <https://doi.org/10.1006/taap.1998.8550>
- Lukacova J, Jambor T, Knazicka Z et al (2015) Dose- and time-dependent effects of bisphenol A on bovine spermatozoa in vitro. *J Environ Sci Heal Part A* 50:669–676. <https://doi.org/10.1080/10934529.2015.1011963>
- MacLeod DJ, Sharpe RM, Welsh M et al (2010) Androgen action in the masculinization programming window and development of male reproductive organs. *Int J Androl* 33:279–287. <https://doi.org/10.1111/j.1365-2605.2009.01005.x>
- Main KM, Mortensen GK, Kaleva MM et al (2006) Human breast milk contamination with phthalates and alterations of endogenous reproductive hormones in infants three months of age. *Environ Health Perspect* 114:270–276. <https://doi.org/10.1289/ehp.8075>
- National Research Council (US) Committee on the Health Risks of Phthalates (2008) National Research Council (US) Committee on the Health Risks of Phthalates. Phthalates and Cumulative Risk Assessment: The Tasks Ahead. National Academies Press (US), Washington (DC)
- Oishi S, Hiraga K (1980) Testicular atrophy induced by phthalic acid esters: effect on testosterone and zinc concentrations. *Toxicol Appl Pharmacol* 53:35–41. [https://doi.org/10.1016/0041-008x\(80\)90378-6](https://doi.org/10.1016/0041-008x(80)90378-6)
- Oskam IC, Lyche JL, Krogenæs A et al (2005) Effects of long-term maternal exposure to low doses of PCB126 and PCB153 on the reproductive system and related hormones of young male goats. *Reproduction* 130:731–742. <https://doi.org/10.1530/rep.1.00690>
- Pant N, Pant A, Shukla M et al (2010) Environmental and experimental exposure of phthalate esters: the toxicological consequence on human sperm. *Hum Exp Toxicol* 30:507–514. <https://doi.org/10.1177/0960327110374205>
- Parmar D, Srivastava S, Seth P (1988) Effect of di(2-ethylhexyl)phthalate (DEHP) on hepatic mixed function

- oxidases in different animal species. *Toxicol Lett* 40:209–217. [https://doi.org/10.1016/0378-4274\(88\)90043-4](https://doi.org/10.1016/0378-4274(88)90043-4)
- Pocar P, Fiandanesi N, Secchi C et al (2012) Exposure to Di(2-ethyl-hexyl) phthalate (DEHP) in utero and during lactation causes long-term pituitary-gonadal axis disruption in male and female mouse offspring. *Endocrinology* 153:937–948. <https://doi.org/10.1210/en.2011-1450>
- Rozati R, Reddy PP, Reddanna P, Mujtaba R (2002) Role of environmental estrogens in the deterioration of male factor fertility. *Fertil Steril* 78:1187–1194. [https://doi.org/10.1016/s0015-0282\(02\)04389-3](https://doi.org/10.1016/s0015-0282(02)04389-3)
- Saillenfait AM, Payan JP, Fabry JP et al (1998) Assessment of the developmental toxicity, metabolism, and placental transfer of Di-*n*-butyl phthalate administered to pregnant rats. *Toxicol Sci* 45:212–224. <https://doi.org/10.1093/toxsci/45.2.212>
- Sathyanarayana S, Karr CJ, Lozano P et al (2008) Baby care products: possible sources of infant phthalate exposure. *Pediatrics* 121:e260–e268. <https://doi.org/10.1542/peds.2006-3766>
- Sharpe RM, Auharek S, Scott HM, de Franca LR, Drake AJ, van den Driesche S (2011) Origin of testicular dysgenesis syndrome disorders in the Masculinization programming Window: relevance to final testis size (=Sperm Production). In: Bourguignon JP, Jégou B, Kerdellhué B, Toppari J, Christen Y (eds) *Multi-system endocrine disruption. Research and perspectives in endocrine interactions*. Springer, Berlin, Heidelberg
- Silva MJ, Reidy JA, Herbert AR et al (2004) Detection of phthalate metabolites in human amniotic fluid. *Bull Environ Contam Toxicol*. <https://doi.org/10.1007/s00128-004-0374-4>
- Singleton DW, Feng Y, Chen Y et al (2004) Bisphenol-A and estradiol exert novel gene regulation in human MCF-7 derived breast cancer cells. *Mol Cell Endocrinol* 221:47–55. <https://doi.org/10.1016/j.mce.2004.04.010>
- Skakkebaek NE, Rajpert-De Meyts E, Main KM (2001) Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects: opinion. *Hum Reprod* 16:972–978. <https://doi.org/10.1093/humrep/16.5.972>
- Skakkebaek NE, Rajpert-De Meyts E, Buck Louis GM et al (2016) Male reproductive disorders and fertility trends: influences of environment and genetic susceptibility. *Physiol Rev* 96:55–97. <https://doi.org/10.1152/physrev.00017.2015>
- The Danish Environmental Protection Agency (2013) *Phthalate strategy*. The Danish Environmental Protection Agency, Copenhagen
- Vitku J, Heracek J, Sosvorova L et al (2016) Associations of bisphenol A and polychlorinated biphenyls with spermatogenesis and steroidogenesis in two biological fluids from men attending an infertility clinic. *Environ Int* 89–90:166–173. <https://doi.org/10.1016/j.envint.2016.01.021>
- Wan HT, Mruk DD, Wong CKC, Cheng CY (2013) Targeting testis-specific proteins to inhibit spermatogenesis: lesson from endocrine disrupting chemicals. *Expert Opin Ther Targets* 17:839–855. <https://doi.org/10.1517/14728222.2013.791679>
- Wang S-W, Wang SS-W, Wu D-C et al (2013) Androgen receptor-mediated apoptosis in bovine testicular induced pluripotent stem cells in response to phthalate esters. *Cell Death Dis* 4:e907. <https://doi.org/10.1038/cddis.2013.420>
- Wang Y-X, You L, Zeng Q et al (2015) Phthalate exposure and human semen quality: results from an infertility clinic in China. *Environ Res* 142:1–9. <https://doi.org/10.1016/j.envres.2015.06.010>
- Welsh M, Saunders PTK, Fisk M et al (2008) Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. *J Clin Invest* 118:1479–1490. <https://doi.org/10.1172/jci34241>
- Wooten KJ, Smith PN (2013) Canine toys and training devices as sources of exposure to phthalates and bisphenol A: quantitation of chemicals in leachate and in vitro screening for endocrine activity. *Chemosphere* 93:2245–2253. <https://doi.org/10.1016/j.chemosphere.2013.07.075>
- Wu W, Zhou F, Wang Y et al (2017) Phthalate levels and related factors in children aged 6–12 years. *Environ Pollut* 220:990–996. <https://doi.org/10.1016/j.envpol.2016.11.049>
- Ye L, Zhao B, Hu G et al (2011) Inhibition of human and rat testicular steroidogenic enzyme activities by bisphenol A. *Toxicol Lett* 207:137–142. <https://doi.org/10.1016/j.toxlet.2011.09.001>
- Yurdakok B, Tekin K, Daskin A, Filazi A (2015) Effects of polychlorinated biphenyls 28, 30 and 118 on bovine spermatozoa in vitro. *Reprod Domest Anim* 50:41–47. <https://doi.org/10.1111/rda.12447>
- Yurdakok-Dikmen B, Stelletta C, Tekin K, et al (2017) Effects of phthalates on bovine primary testicular and epididimal cells in vitro. In: *56th Annual Meeting and ToxExpo*. Baltimore, Maryland, USA. Abstract Book p 47
- Yurdakok-Dikmen B, Stelletta C, Tekin K et al (2019) Effects of phthalates on bovine primary testicular culture and spermatozoa. *Cytotechnology* 71:935–947. <https://doi.org/10.1007/s10616-019-00336-z>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.