Received: 25 June 2022 | Accepted: 29 December 2022

DOI: 10.1111/ahe.12902

Anatomia Histologia Embryologia

WILEY

ORIGINAL ARTICLE

Effect of di(*n*-butyl) phthalate on the blood–testis barrier during puberty onset

Reneilwe A. Molele[1] | Musa Zakariah[2,3] | Mohammed I. A. Ibrahim[4,5] | Mohamed A. A. Mahdy[6,7] | Geoffrey T. Fosgate[1] | Geoffrey Brown[1]

[1]Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa

[2]Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa

[3]Department of Veterinary Anatomy, Faculty of Veterinary Medicine, PMB 1069 University of Maiduguri, Maiduguri, Nigeria

[4]Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa

[5]Department of Basic Science, University of West Kordofan, Al-Fulah, Sudan

[6]Department of Anatomy and Embryology, Faculty of Veterinary Medicine, South Valley University, Qena, Egypt

[7]Department of Anatomy and Histology, Faculty of Veterinary Medicine, King Salman International University, Ras Sudr, Egypt

Correspondence
Reneilwe A. Molele, Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Private bag X04, Onderstepoort, Pretoria 0110, South Africa.
Email: reneilwearetha@gmail.com

Funding information
National Research Foundation

Abstract

Di(*n*-butyl) phthalate (DBP) is considered a substance of serious concern because of its reproductive toxicity and endocrine-disrupting properties. Exposure to DBP causes morphological and functional changes in the male reproductive system of birds and mammals. However, there are no detailed reports on the effects of DBP on the Sertoli cell and junctional complexes of the blood–testis barrier (BTB) in birds. The present study investigated dose-related ultrastructural changes in Sertoli cells and junctional complexes of the BTB in adult Japanese quail (*Coturnix coturnix japonica*) exposed to DBP prior to puberty. A total of 25 Japanese quail were used for the study. Exposure to DBP doses of 50, 200 and 400 mg DBP/kg/d caused dose-related ultrastructural changes in junctional complexes including dilation and separation, while disruption of cytoplasmic membranes and mitochondria was observed in Sertoli cells. There was a significant difference in the sum of vacuoles, vacuole diameter, nuclear width, nuclear length, nuclear area, sum of damaged spherical mitochondria, width of elongated mitochondria and the sum of damaged elongated mitochondria among the five treatment groups ($p < 0.05$). Prepubertal exposure to DBP at doses of 50, 200 and 400 mg DBP/kg/d for 30 days led to adverse effects in the adult male Japanese quail reproductive system by inducing structural changes in the Sertoli cells and junctional complexes. Such changes might disrupt the BTB and potentially interfere with spermatogenesis. Results indicated that the Sertoli cell is sensitive to DBP exposure and might be an important cellular target for DBP-induced testicular toxicity.

KEYWORDS
blood–testis barrier, di(*n*-butyl)phthalate, mitochondrial disruption, Sertoli cells, testicular toxicity

1 | INTRODUCTION

Phthalates are widely distributed industrial chemicals that are used as plasticizers to soften polyvinyl chloride (PVC)-based products (Hernández-Díaz et al., 2009). When used as plasticizers, phthalates do not bind permanently to the products to which they are added and might be released into the surrounding environment during production and disposal of PVC and other phthalate-containing products (Clara et al., 2010; Fierens et al., 2012; Heudorf et al., 2007; Staples et al., 1997). Phthalates are an important group of endocrine-disruptor chemicals because of their anti-androgenic and oestrogen-like activity (Akingbemi et al., 2004;Skinner, 2016). Thus, these chemicals are reproductive, as well as developmental toxicants (Akingbemi et al., 2001;Hannon & Flaws, 2015). Exposure to phthalates transpires by intake of contaminated food, contact through the skin and inhalation of contaminated air (Heudorf et al., 2007; Olujimi

et al., 2010). Previous studies have shown that phthalate exposure causes morphological and functional alterations in the male reproductive system of birds and mammals, with the Sertoli cell being the main cellular target for toxicants (Bao et al., 2011; Bello et al., 2019; Ibrahim et al., 2021; Kumar et al., 2015; Monsees et al., 2000).

Of the various phthalate compounds, di(*n*-butyl) phthalate (DBP) is considered one of the most significant since it is utilized extensively in industrial manufacturing. It is considered a substance of very serious concern because of its reproductive toxicity and endocrine-disrupting properties (Wittassek & Angerer, 2008). Food and water contamination with DBP has been reported in several countries including South Africa, Taiwan and the United Kingdom (Bradley et al., 2013; Olujimi et al., 2010; Yuan et al., 2002). Experimental evidence has shown that high levels of DBP cause toxic effects on testicular function, thereby causing a decrease in testosterone concentrations as reported in men working in a factory producing PVC flooring and potentially exposed to DBP (Pan et al., 2006). Previous reports have demonstrated ultrastructural changes in the Sertoli cell cytoplasm, and disruption of Sertoli cell vimentin filaments of prepubertal (3-week-old) rats exposed to 500 mg/kg DBP (Alam, Andrina, et al., 2010; Alam, Ohsako, et al., 2010). In addition, dose-related degeneration of Sertoli cells has been reported in Wistar rats following exposure to 500, 1000 and 1500 mg/kg DBP (Nair, 2015).

Sertoli cells support the developing germ cells structurally and nutritionally and form the blood–testis barrier (BTB) by various junctional complexes (Sharma et al., 2018; Sikka & Wang, 2008). Junctional complexes include, but are not limited to adherens, gap and tight junctions, and are comprised of specialized proteins involved in cell adhesion, communication and attachment, respectively (Ahmed et al., 2018). Adherens junctions are responsible for the lateral adhesions between Sertoli and germ cells (Yan et al., 2009). Gap junctional communication has an important function in testis development and sperm maturation, while tight junctions constitute a major part of the BTB (Kidder & Cyr, 2016).

Long-term adverse effects have been demonstrated in adult mice exposed prepubertally indicating the sensitivity of prepubertal mice testes (Moody et al., 2013). In addition, prepubertal mice testes had impaired Sertoli cell maturation and delayed spermatogenesis, even after exposure to low DBP (10 mg/kg/d) concentrations (Moody et al., 2013). Laboratory studies have also demonstrated that the male Japanese quail is sensitive to DBP exposure during development (Monsees et al., 2000; Ottinger et al., 2002). Recently, Ibrahim et al. (2021) demonstrated that exposure of Japanese quail to DBP prior to the onset of puberty causes histopathological changes in the epididymal epithelium in a dose-related manner. Histological effects included apoptosis, cytoplasmic vacuoles and autophagy (Ibrahim et al., 2021). Exposure to DBP also alters the morphology of seminiferous tubule and impairs Leydig cell steroidogenesis (Bello et al., 2014; Zakariah et al., 2022). Although studies on the reproductive toxicological effects resulting from exposure to DBP have been conducted in the male Japanese quail (Bello et al., 2014, 2019; Ibrahim et al., 2021), there are no detailed reports on the effects of environmentally relevant concentrations of DBP on the Sertoli cell and junctional complexes of the BTB in the species. According to Tyler et al. (2018), environmentally relevant concentrations of DBP are those in the low μg/L range, while Liu and Craig (2019) classified environmentally relevant levels of DBP as those between 10 and 100 μg/kg/day.

The Japanese quail is a common laboratory species for environmental, behavioural, developmental and comparative studies (Ainsworth et al., 2010; Zakariah et al., 2020). Quail are considered as the representative of terrestrial birds and are recognized models for evaluation of the effects of toxicants in wild birds (Environmental Protection Agency, 1996). Furthermore, the quail is considered an ideal biological and experimental model due to its rapid growth rate and is commonly utilized in reproductive toxicity studies (Bello et al., 2014; Ibrahim et al., 2021; Zakariah et al., 2022). Japanese quail have an advanced neuroendocrine system that shares basic characteristics with mammals (Ball & Balthazart, 2010). Therefore, the quail was considered an appropriate model to investigate dose-related morphological changes in Sertoli cells and junctional complexes of the BTB in adult Japanese quail exposed to DBP during the prepubertal stage.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

The study was approved by the University of Pretoria's Animal Ethics Committee (Approval number A065-12). All procedures were performed according to the South African Bureau of Standards (SABS) guidelines for the Care and Use of Laboratory and Research Animals (SABS, 2008). The experiment was carried out according to the guidelines for avian toxicity testing studies as specified by the Organization for Economic Co-operation and Development (OECD, 2010).

2.2 | Chemicals used for the study

Di(*n*-butyl) phthalate (CAS Number 84-74-2 technical grade, purity > 99.8%, PN61840625001730) was purchased from Sigma Aldrich (Pty) Ltd (Johannesburg, South Africa).

2.3 | Animals and management

A total of 25 newly hatched, male Japanese quail procured from the Aviary Unit, Irene Animal Improvement Research Station, Pretoria, were used for the study. At hatching, the environmental temperature was maintained at 35–37°C and then slowly decreased by 0.5°C/day until a temperature of 16°C–23°C was reached at 4 weeks of age. Thereafter, the birds, which were housed at the Poultry Research Unit of the Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, South

Africa, were maintained in battery cages (46 × 95 × 51 cm) under a controlled photoperiod (16 h light: 8 h dark), and temperature (25°C ± 2°C) with a relative humidity of 50% ± 5%. Birds were individually identified by means of wing tags and had access to a standard commercial high-protein poultry diet (Obaro Feeds®, Pretoria, South Africa), and freshwater ad libitum throughout the experiment.

2.4 | Animal exposure and dosing regimen

At 4 weeks of age, birds were randomly divided into five dosage groups ($n = 5$ per group). Control birds were administered a corn-oil vehicle only (a dose of 1 ml/kg/d), while the other four experimental groups received a daily dosage of 10, 50, 200 and 400 mg/kg body weight of DBP (dissolved in corn oil) intragastrically, respectively, for a period of 30 days. The same doses (10, 50, 200 and 400 mg DBP kg/d) were used by Bello et al. (2014) and Ibrahim et al. (2021) to investigate the effect of DBP on Leydig cells, and on the morphology of the epididymal region, respectively, in the Japanese quail.

2.5 | Sample collection

Quail were euthanized by carbon dioxide inhalation on the last day (Day 30) of the experimental period. Testicular tissue samples (~1 mm^3 blocks) were collected and immediately fixed in 4% glutaraldehyde in 0.13 M Millonig's phosphate buffer (pH 7.4), for at least 24 h.

2.6 | Transmission electron microscopy

Tissue samples were postfixed in 1% osmium tetroxide for 2 h, dehydrated in an ascending series of alcohol concentrations and embedded in epoxy: resin at a ratio of 1:2 for 1 h, 1:1 for 2 h and 100% resin overnight. Semithin sections (1 μm thick) were cut and stained with toluidine blue. Stained sections were examined and photographed using an Olympus BX-63 microscope equipped with a DP72 camera and CellSens® dimension (Olympus Corporation, Tokyo, Japan) imaging software. For transmission electron microscopy (TEM), ultrathin sections (50–90 nm thick) were cut, mounted on copper grids and double stained with lead acetate and counterstained with uranyl citrate. Grids were viewed under a Phillips CM10 transmission electron microscope (FEI, Hillsboro, The Netherlands) and equipped with an Olympus Megaview III imaging system.

Linear measurements were carried out on TEM images at the magnification of 2 μm using ImageJ software version 1.52 (National Institutes of Health, Bethesda, Maryland, USA). The vacuole diameter, nuclear width, nuclear length and nuclear area, diameter of spherical mitochondria, width, length and area of elongated mitochondria were measured. Six fields were evaluated per bird ($n = 5$ birds per group) and averages of all measurements were recorded. In addition, the sum of vacuoles, normal and damaged spherical, and elongated mitochondria were recorded in the same fields.

2.7 | Statistical analysis

IBM SPSS software version 27 (IBM Corp., Armonk. NY) was utilized for statistical analysis, and data were presented as median (range). The normality assumption was assessed by calculating descriptive statistics, plotting histograms and performing the Shapiro–Wilk normality test. The Kruskal–Wallis test was utilized for the comparison of the distribution of parameters across DBP treatment groups followed by pairwise Mann–Whitney *U* tests with Bonferroni correction of *p*-values for multiple post hoc tests. The differences were considered significant when $p \leq 0.05$.

3 | RESULTS

3.1 | The effect of DBP on Sertoli cell

Histologically, in the control and DBP-treated groups, Sertoli cells were irregularly shaped and comprised an extensive cytoplasm (Figure 1a–d). No morphological changes were detected in Sertoli cells of quail treated with 10 mg DBP/kg/d. Sertoli cells of quail treated with 50 mg DBP/kg/d and had noticeable cytoplasmic vacuolation and slight cytoplasmic shrinkage in comparison with the control group (Figure 1b). However, quail treated with 200 and 400 mg DBP/kg/d displayed marked histopathological changes in the Sertoli cells including cytoplasmic shrinkage, cellular degeneration, disruption of cytoplasmic membranes and detachment of the cells from the basement membranes (Figure 1c,d). In addition, there were more frequent intercellular spaces at Sertoli cell–Sertoli cell contacts and at Sertoli cell–germ cell contacts in comparison with the control group (Figure 1d).

Ultrastructurally, there were some differences in the cytoplasm of Sertoli cells treated with 50 mg DBP/kg/d from the control group (Figure 2a–c). Disruption of mitochondria characterized by loss of cristae dilated rough endoplasmic reticulum, and shrinkage and degeneration of the nucleus were observed in Sertoli cells of birds treated with 50 mg DBP/kg/d (Figure 2b,c). Sertoli cells of quail treated with 200 mg DBP/kg/d displayed an increase in size of cytoplasmic vacuoles, distorted mitochondria with denser cristae, nuclear shrinkage and dilation of the rough endoplasmic reticulum (Figure 2d,e). Administration of 400 mg DBP/kg/d induced Sertoli cell degeneration characterized by chromatin condensation, nuclear shrinkage, highly dilated rough endoplasmic reticulum, nuclear invagination and mitochondrial disruption (Figure 2f,g).

Histometrically, there was a significant difference in the sum of vacuoles, vacuole diameter, nuclear width, length, area, sum of damaged spherical mitochondria, width of elongated mitochondria and the sum of damaged elongated mitochondria among the five treatment groups ($p < 0.05$; Table 1). The sum of vacuoles, vacuole diameter, sum of damaged spherical and elongated mitochondria were significantly ($p \leq 0.001$) different among doses, with the 400 mg DBP/kg/d-treated group displaying the highest median scores (Table 1).

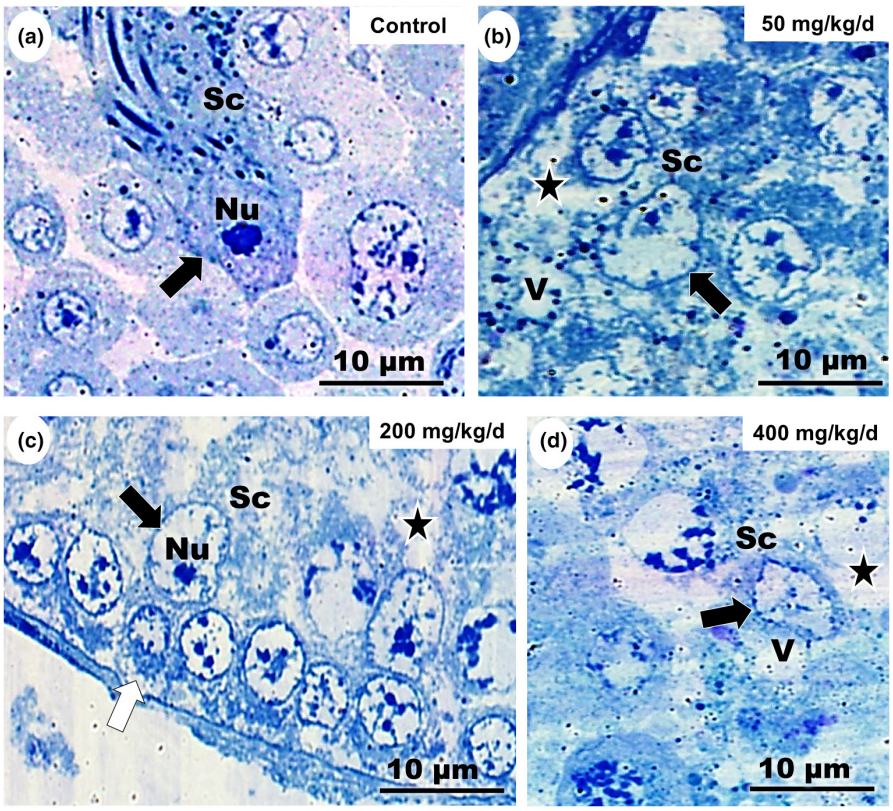

FIGURE 1 Light micrographs of Sertoli cells in Japanese quail testes. (a) Control, (b) 50, (c) 200 and (d) 400 mg DBP/kg/d. (a) Normal Sertoli cells (Sc). (b) Vacuoles (V) were observed in the cytoplasm of Sc. Note the cytoplasmic change in the Sc. (c) Dissolve of the nuclear chromatin, degeneration, cytoplasmic shrinkage and disrupted cytoplasmic membrane in Sc. Note the detachment of Sc from the basement membrane (white arrow). (d) Degenerative Sc characterized by shrinkage of cytoplasm and nucleus (arrow). Note the intercellular spaces (stars) at Sertoli cell–Sertoli cell and Sertoli cell–spermatogenic cell contacts indicating degeneration. Black arrows: Sertoli cell nucleus, white arrow: detached Sc from the basement membranes, Nu: Sertoli cell nucleolus, V: vacuoles, star: intercellular spaces between adjacent cells.

3.2 | The effect of DBP treatment on junctional complexes

In all studied groups, adherens junctions were observed at Sertoli cell–spermatocyte, Sertoli cell–spermatogonia and Sertoli cell–Sertoli cell contacts (Figure 3a–d). Gap junctions were present at Sertoli cell–spermatogonia, Sertoli cell–Sertoli cell and Sertoli cell–spermatocyte contacts (Figure 4a–d), while tight junctions were observed at Sertoli cell–Sertoli cell contacts (Figure 5a–d).

In the control and treated groups, junctional complexes were well-developed and sites of membrane apposition between adjacent cells were clearly defined (Figures 3–5a–d). Junctional complexes in treated groups were generally electron-dense when compared to the control group (Figures 3–5a–d). Relative to controls, no major changes were observed in the junctional complexes of birds in the 10 mg DBP/kg/d group, while those in the other DBP-treated groups (50, 200 and 400 mg DBP/kg/d) were more electron-dense (Figures 3 and 4b–d) and appeared dilated and separated (Figure 5b–d).

4 | DISCUSSION

Since Sertoli cells play a crucial role of providing structural and nutritional support for developing germ cells, any toxic agent that causes injury to the Sertoli cell might cause abnormal spermatogenesis (Chang et al., 2017; Mruk & Cheng, 2004). In the present study, no histopathological and ultrastructural changes were observed in the Sertoli cells of the 10 mg DBP/kg/d-treated group. Similarly, previous reports reported that Japanese quail exposed to 10 mg DBP/kg/d showed no morphological changes in the testes (Bello et al., 2014; Zakariah et al., 2022) and epididymal region (Ibrahim et al., 2021), while those exposed to 50 mg DBP/kg/d showed minor changes (Bello et al., 2014; Ibrahim et al., 2021; Zakariah et al., 2022).

The current study demonstrated that DBP concentrations of 50, 200 and 400 mg DBP/kg/d induced mitochondrial disruption, dilation of rough endoplasmic reticulum and nuclear shrinkage. Previous studies in the Japanese quail have reported considerable evidence that DBP doses of 200 and 400 mg/kg/d induced different reproductive effects in the species. The effects include changes in the architecture of the testes as evidenced by poor testicular development and altered spermatogenesis (Bello et al., 2014).

The same concentrations (200 and 400 mg/kg/d of DBP) have been shown to induce loss of normal architecture in the proximal efferent duct epithelium. At the ultrastructural level, ciliated cells with multiple nuclei have been reported in the epithelium of the distal efferent ductules of the Japanese quail (Ibrahim et al., 2021). In rats, no histopathological changes were observed in the testes following exposure to 250 mg DBP/kg/d, suggesting that rats are less sensitive to DBP exposure than Japanese quail. However, significant histopathological changes including degeneration of seminiferous tubule and decreased spermatogenic cells were observed in rats treated with 500 mg/kg/d DBP (Zhou et al., 2010).

Di(*n*-butyl) phthalate has also been reported to decrease the levels of follicle-stimulating hormone (FSH), testosterone and luteinizing hormone (LH), resulting in low sperm count and motility, and increased percentage of abnormal sperm in the testes

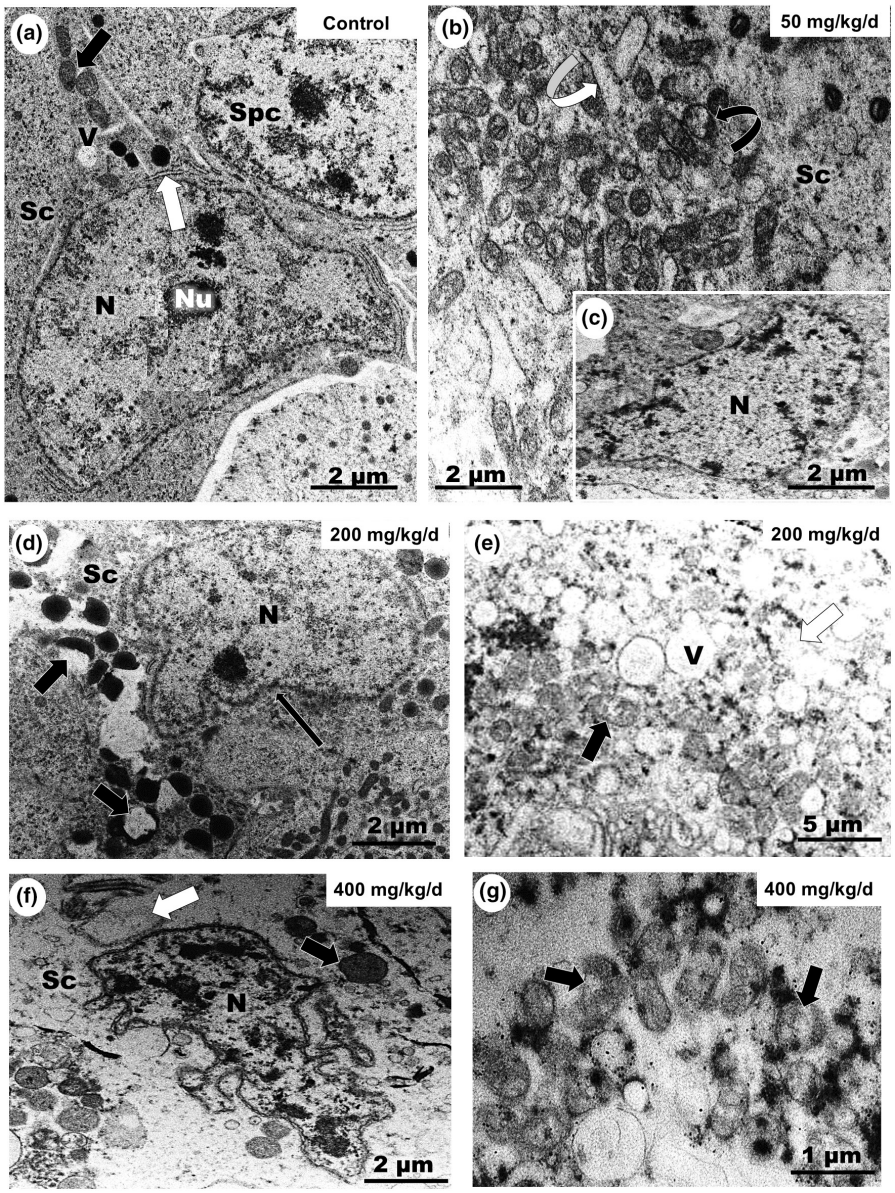

FIGURE 2 Electron micrographs of Sertoli cells (Sc) in Japanese quail testes. (a) Control, (b,c) 50-, (d,e) 200- and (f,g) 400 mg DBP/kg/d. (a) The cytoplasm of Sc showed normal ultrastructural appearance with large, irregularly shaped nuclei (N), rough endoplasmic reticulum (thick/broad white arrow) and mitochondria (thick/broad black arrow) with evident tubular cristae. (b,c) Disruption of mitochondria characterized by loss of cristae (curved black arrow), dilated rough endoplasmic reticulum (curved white arrow), as well as shrinkage and degeneration of the nucleus (N). (d,e) Sc mitochondria showed cristae distortion with increased density (thick black arrow), as well as vacuoles (V) in the cytoplasm of the cell. In addition, dilation of rough endoplasmic reticulum (thick white arrow) and nuclear shrinkage (thin black arrow) were observed. (f) Degenerative Sc characterized by nuclear (N) shrinkage and chromatin condensation. Note the dilated rough endoplasmic reticulum (white arrow) and mitochondrial disruption (black arrow) in the Sc cytoplasm. (g) Swollen and disrupted mitochondria displaying loss of cristae (arrow)

(Huan et al., 2011). A study conducted in prepubertal Japanese quail exposed to 500 mg/kg DBP for 3, 6 and 24 h suggested that DBP blocked LH secretion from the hypothalamus and/or pituitary, thereby decreasing LH stimulation of Leydig cells and reducing intratesticular testosterone concentrations (Alam & Kurohmaru, 2021). The study further demonstrated that DBP-induced decreases in intratesticular testosterone concentrations might cause changes to the physical structure of Sertoli cells, which, in turn, might induce germ cell apoptosis in the Japanese quail (Alam & Kurohmaru, 2021). A reduction in cellular testosterone levels was also observed in the Japanese quail, following prepubertal exposure to 200 and 400 mg DBP/kg/d (Bello et al., 2014). However, in prepubertal rats, DBP exposure to doses of 500 and 1000 mg/kg/d caused increased levels of FSH and LH, but reduced testosterone levels (Huan et al., 2011).

The current study demonstrated that there was a decrease in the nuclear widths, as well as the lengths and widths of the elongated mitochondria in the 50, 200 and 400 mg DBP/kg/d-treated groups in comparison with the control group and a previous study in the normal adult Japanese quail (Molele et al., 2021). The findings of the current study also showed that prepubertal exposure of Japanese quail to DBP doses of 50, 200 and 400 mg DBP/kg/d for 30 days, causes vacuolation of the Sertoli cell cytoplasm in a dose-dependent manner. Vacuolation of Sertoli cells is suggested to be the earliest morphological sign of DBP-associated testicular toxicity, whereby DBP causes toxicity by inducing oxidative stress (Alam, Andrina, et al., 2010; Turrens, 2003; Zhou et al., 2010). A previous study demonstrated that treatment of adult rats with DBP at doses of 250 and 500 mg/kg/d induces oxidative stress in the testes by decreasing the activities of anti-oxidant enzymes and increasing lipid peroxidation (Zhou et al., 2010).

The increased intercellular spaces observed at Sertoli cell–Sertoli cell and Sertoli cell–germ cell contacts in quail exposed to 200 and 400 mg DBP/kg/d indicate the location of germ cell degeneration caused by a compromised BTB (Levy et al., 1999). In addition, this

TABLE 1 Sertoli cell measurements [median (min-max)] of the adult Japanese quail following treatment with di(n-butyl) phthalate (DBP) at doses 10, 50, 200 and 400 mg/kg/d during puberty onset

Parameter	Control	10 mg DBP/kg/d	50 mg DBP/kg/d	200 mg DBP/kg/d	400 mg DBP/kg/d	*p* Value*
Sum of vacuoles	34 (26–40)^{a}	36 (30–38)^{a}	39 (34–41)^{ab}	48 (44–51)^{ab}	50 (47–57)^{b}	**$p = 0.001$**
Vacuole diameter	0.33 (0–0)^{a}	0.37 (0–0)^{ab}	0.48 (0–1)^{abc}	0.55 (0–1)^{bc}	0.66 (1–1)^{c}	**$p < 0.001$**
Nuclear width	5.376 (5.029–5.812)^{a}	4.392 (3.245–6.118)^{ab}	4.138 (2.004–5.093)^{ab}	4.178 (2.331–4.644)^{ab}	2.817 (2.342–3.674)^{b}	**$p = 0.011$**
Nuclear length	6.897 (6.720–8.803)^{a}	6.024 (5.864–7.610)^{ab}	6.039 (3.617–7.506)^{ab}	5.888 (5.601–7.210)^{ab}	5.251 (4.208–5.725)^{b}	**$p = 0.024$**
Nuclear area	40.123 (35.453–49.833)^{a}	27.069 (20.571–47.821)^{abc}	35.422 (23.383–83.373)^{ab}	24.867 (14.061–33.531)^{abc}	13.319 (11.291–19.812)^{c}	**$p = 0.003$**
Sum of spherical mitochondria	76 (66–79)^{a}	72 (65–82)^{a}	73 (59–104)^{a}	74 (69–93)^{a}	83 (79–87)^{a}	$p = 0.221$
Diameter of spherical mitochondria	0.315 (0.219–0.539)^{a}	0.348 (0.223–0.545)^{a}	0.272 (0.241–0.539)^{a}	0.371 (0.354–0.442)^{a}	0.331 (0.176–0.359)^{a}	$p = 0.338$
Sum of damaged spherical mitochondria	0 (0–0)^{a}	0.018 (0.011–0.045)^{ab}	0.168 (0.076–0.263)^{abc}	0.429 (0.305–0.479)^{bc}	0.626 (0.577–0.689)^{c}	**$p < 0.001$**
Sum of elongated mitochondria	74 (66–84)^{a}	70 (63–86)^{a}	76 (67–87)^{a}	88 (56–91)^{a}	84 (70–99)^{a}	$p = 0.431$
Width of elongated mitochondria	0.256 (0.235–0.266)^{a}	0.273 (0.229–0.285)^{a}	0.245 (0.224–0.275)^{ab}	0.221 (0.184–0.247)^{ab}	0.191 (0.187–0.218)^{b}	**$p = 0.005$**
Length of elongated mitochondria	1.073 (0.822–1.344)^{a}	0.850 (0.787–0.905)^{a}	1.029 (0.811–1.226)^{a}	1.027 (0.934–1.169)^{a}	1.079 (0.783–1.324)^{a}	$p = 0.162$
Area of elongated mitochondria	0.282 (0.195–0.355)^{a}	0.232 (0.179–0.254)^{a}	0.253 (0.179–0.338)^{a}	0.232 (0.171–0.274)^{a}	0.203 (0.150–0.254)^{a}	$p = 0.459$
Sum of damaged elongated mitochondria	0 (0–0)^{a}	0.034 (0–0.053)^{ab}	0.139 (0.057–0.248)^{abc}	0.379 (0.303–0.457)^{bc}	0.668 (0.604–0.728)^{c}	**$p < 0.001$**

Note: Values represent median (min-max), $n = 5$ per group. Different superscripts in a column indicate a significant difference across the groups ($p \leq 0.05$). The bolded values are ≤0.05 and indicate where there was a significant difference in the parameters between the different dosage groups.

*Based on Kruskal–Wallis tests comparing parameters among all five treatment groups.

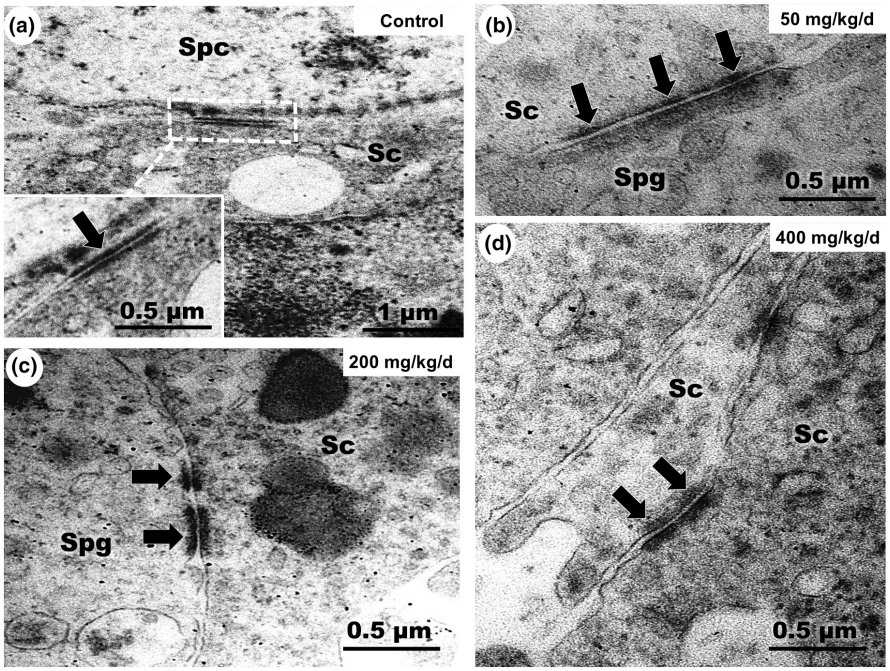

FIGURE 3 Electron micrographs of adherens junctions in Japanese quail testes. (a) Control, (b) 50, (c) 200 and (d) 400 mg DBP/kg/d. (a) Normal adherens junctions in testis (arrow). (b–d) Electron-dense cytoplasmic membranes where junctions are formed. Arrows show the discontinuous adherens junctions. Spc, spermatocyte; Sc, Sertoli cell; Spg, spermatogonium

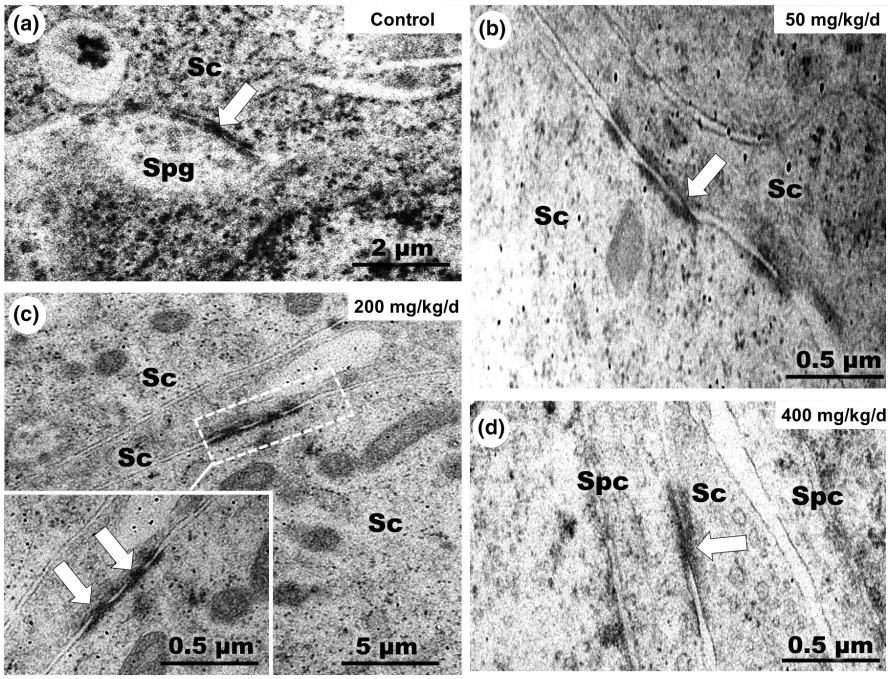

FIGURE 4 Electron micrographs of gap junctions in Japanese quail testes. (a) Control, (b) 50, (c) 200 and (d) 400 mg DBP/kg/d. (a) Arrow indicates normal gap junction between a Sertoli cell and spermatogonia. (b–d) Arrows show electron-dense gap junctions. Sc, Sertoli cell; Spg, spermatogonium; Spc, spermatocyte

suggests that, in the Japanese quail, the effects of DBP on the BTB might be evident after exposure to concentrations of 200 mg DBP/kg/d or greater.

Sertoli cells are responsible for the formation of the BTB by means of junctional complexes including adherens, gap and tight junctions (Sharma et al., 2018; Sikka & Wang, 2008). The findings of the present study indicate that the junctions in quail treated with 50, 200 and 400 mg DBP/kg/d were altered, suggesting a disruption of junctional complexes and the BTB (Hu et al., 2014). Although the expression of junctional proteins was not assessed, previous research suggests that toxicants lead to a decrease or redistribution in junctional proteins, thereby disrupting the BTB (Chang et al., 2017). Concurring with the present findings, dilatation and separations of junctional complexes have been reported in mice treated with melamine at doses of 100 and 500 µg/ml for 24 h (Chang et al., 2017). The findings of the present study are limited by the relatively small sample size ($n = 5$ per treatment group), which reduces the precision of our observations and estimates. Additionally, it would have been advantageous to measure concentrations of FSH, LH, testosterone, and inhibin and to carry out immunohistochemical analyses for activin type II receptors (ActRIIa and ActRIIb) in Sertoli cells to complement the morphological and morphometric data. Furthermore, there

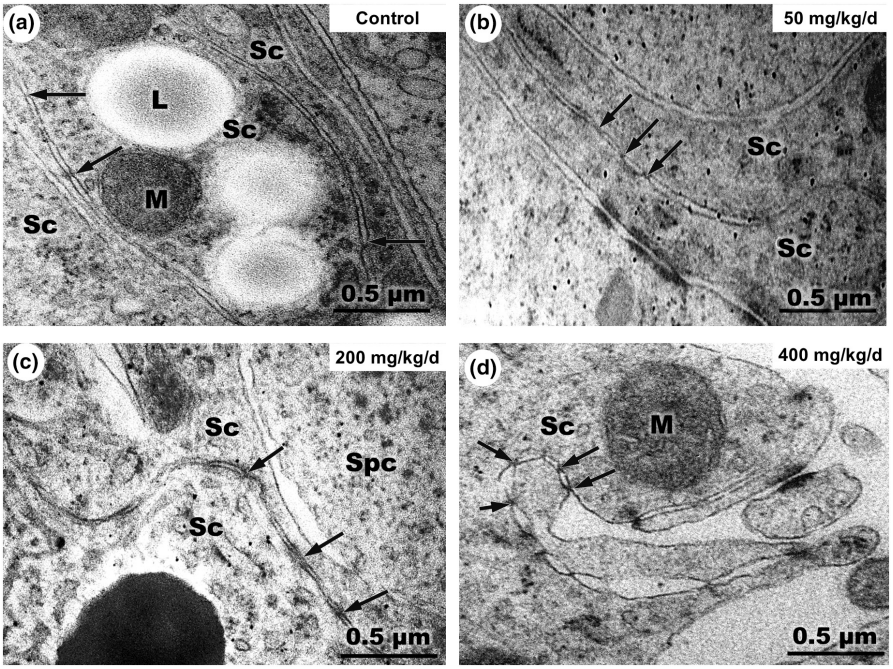

FIGURE 5 Electron micrographs of tight junctions in Japanese quail testes. (a) Control, (b) 50, (c) 200 and (d) 400 mg DBP/kg/d. (a) Normal tight junctions (arrows) between adjacent Sertoli cells. (b–d) Different degrees of dilatation and separation in the tight junctions between adjacent Sertoli cells. Sc, Sertoli cell; L, lipid droplets; M, mitochondrion; Spc, spermatocyte

is a need to investigate the expression of the proteins associated with the junctional complexes under investigation.

In conclusion, the current study demonstrated that prepubertal exposure to DBP doses of 50, 200 and 400 mg DBP/kg/d for 30 days causes adverse effects in the adult male Japanese quail reproductive system by inducing structural changes in the Sertoli cells and junctional complexes of the BTB in a dose-dependent manner. Moreover, the current findings revealed that the male Japanese quail is sensitive to DBP exposure and that alterations in the Sertoli cells and junctional complexes might consecutively lead to a disruption of the BTB and potentially interfere with the process of spermatogenesis. Our observations further suggest that the Japanese quail Sertoli cell is sensitive to DBP exposure and might be the main cellular target and therefore a good biomarker for monitoring and assessing DBP-induced male reproductive toxicity.

ACKNOWLEDGEMENTS

The authors thank Antoinette Lensink and Lizette du Plessis of the Electron Microscopy Unit, Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria, South Africa for their technical support. The study was funded by the National Research Foundation (NRF) of South Africa (grant #N01521). The University of Pretoria is acknowledged for the PhD postgraduate bursary awarded to Reneilwe Molele.

CONFLICT OF INTEREST

There is no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Reneilwe A. Molele https://orcid.org/0000-0001-7905-9758
Musa Zakariah https://orcid.org/0000-0002-6285-105X
Mohammed I. A. Ibrahim https://orcid.org/0000-0003-0830-2209
Mohamed A. A. Mahdy https://orcid.org/0000-0001-6402-2944
Geoffrey T. Fosgate https://orcid.org/0000-0002-9432-0042
Geoffrey Brown https://orcid.org/0000-0002-2850-8303

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How to cite this article: Molele, R. A., Zakariah, M., Ibrahim, M. I. A., Mahdy, M. A. A., Fosgate, G. T., & Brown, G. (2023). Effect of di(*n*-butyl) phthalate on the blood–testis barrier during puberty onset. *Anatomia, Histologia, Embryologia*, *52*, 411–420. https://doi.org/10.1111/ahe.12902