Dose-related morphological changes in the epididymal region of sexually active adult male Japanese quail treated with di-*n*-butyl phthalate (DBP) commencing during the pre-pubertal stage

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Highlights

- Di-*n*-butyl phthalate (DBP) induces dose-dependent toxicity in quail's epididymis.
- The proximal efferent ductule is more sensitive to the effects of the DBP.
- Oligospermia was observed in the lumen of epididymal duct of adult Japanese quails.
- Pre-pubertal exposure to DBP could cause infertility in the adult Japanese quails.

Abstract

Di-*n*-butyl phthalate (DBP) is widely used as a plasticizer in personal care and medical products and is known to induce toxicity in the male reproductive organs in both mammals and birds. In this study, there was investigation of the effects of DBP on the epithelium of the rete testis, proximal, and distal efferent ductules and epididymal duct of adult Japanese quail (*Coturnix japonica*) following treatment with varying doses during the pre-pubertal and peri-pubertal periods. Pre-pubertal quail (*n* = 25) 4 weeks post-hatching were dosed orally with 10, 50, 200 and 400 mg DBP/kg/d, for 30 days and control birds were administered corn-oil only (*n* = 5 per group). Histo-metrically, there was lesser ($P < 0.001$) epithelial heights of the rete testis and efferent ductules in all quail DBP-treated groups, but not in the epididymal duct epithelium. There were no morphological change effects as a result of DBP treatments in the rete testis epithelium, while there were epithelial cytoplasmic vacuoles detected in the distal efferent ductule and epididymal duct of birds treated with 50, 200 and 400 mg DPB/kg/d. There were several lesions, including degenerative changes, cytoplasmic vacuoles, apoptosis and autophagy in the epithelium of the proximal efferent ductule in quail treated with 200 and 400 mg DBP/kg/d. Overall, the results indicate that treatment with DBP during the pre-pubertal period induced dose-dependent histometric and morphological changes in the epithelium of the epididymal region. It is concluded that the proximal efferent ductule was a highly sensitive component of the epididymal tissues of Japanese quail following treatment with DBP during the pre-pubertal period.

Keywords: Di-*n*-butyl phthalate; Efferent ductules; Epididymal duct; Japanese quail; Morphology; Rete testis

1. Introduction

The urbanization and the increase in the world's population have intensified the wide use of industrial products such as phthalic acid esters (PAEs) in personal care and consumer products, food processing, as well as in medical devices (Hauser and Calafat, 2005; Swan, 2008; Bello et al., 2014). Phthalic acid esters eventually contaminate the environment and air, food, water, dust and soil (Ziółkowska and Wyszkowski, 2010; Przybylińska and Wyszkowski, 2016). These environmental chemicals have endocrine-disrupting activity with resultant adverse effects on the human female and male reproductive systems (Foster, 2005; Knez, 2013). One of the most problematic chemicals in this regard is di-*n*-butyl phthalate (DBP). It is not surprising that the European Union classified DBP as a substance of great concern for environmental contamination (Commission Implementing Decision-EU, 2017).

Di-*n*-butyl phthalate, which is used as a plasticizer in polyvinyl chloride polymers (PVC) products (ECHA, 2010) is a ubiquitous environmental pollutant. There has been growing social concern regarding the potent male reproductive toxicity of DBP reported in experimental animals (Mylchreest et al., 1998; Foster et al., 2001; Ryu et al., 2007; Zhou et al., 2010, 2011; Bello et al., 2014; Sahin et al., 2014; Bello et al., 2019), and concentrations detected in the environment and human biological samples (Dobrzynska, 2016). Results from studies indicate DBP has anti-androgenic effects, especially affecting the male reproductive tissues during sensitive stages of foetal development and during the pre-pubertal period in rats (Mylchreest et al., 1998), mice (Moody et al., 2013) and Japanese quail (Bello et al., 2014). Most notable is the disruption of the androgen-signalling pathway, which ultimately increases the incidence of abnormalities in the male reproductive organs of rats (Mylchreest et al., 1999).

Di-*n*-butyl phthalate induces dose-dependent toxicity of testicular tissues and epididymides of post-pubertal Sprague-Dawley rats (Zhou et al., 2010, 2011), as well as of the testes in Japanese quail (Bello et al., 2014, 2019). Tubular atrophy, interstitial vascular hyperemia and luminal oligozoospermia were reported in the epididymis of the post-pubertal Sprague-Dawley rats following treatment with 500 mg DBP/kg/day (Zhou et al., 2011). Mylchreest et al. (1998) reported a diverse array of morphological effects in the male Sprague-Dawley rats, ranging from minimal disruption of epididymal structures to absence of epididymides to ectopic or complete absence of testes following *in utero* treatments with 250, 500 and 750 mg DBP/kg/day. Similarly, there was exfoliation of the stereociliae and a reduction in the height of the epithelial cells of the epididymides and ductus deferens in pre-pubertal Wistar rats following treatment with 500 and 1,000 mg DBP/kg/day for 30 days (Sahin et al., 2014). Remarkable gross and histological changes including seminiferous tubular atrophy and degeneration occurred in the testicular tissue and Sertoli cells of post-pubertal Japanese quail when there were treatments with 200 and 400 mg DBP/kg/day for 30 days during the pre-pubertal period (Bello et al., 2014, 2019).

Although multiple studies reported the effects of DBP in male reproductive organs of mammals and birds (Wine et al., 1997; Mylchreest et al., 1999, 2000; Barlow and Foster, 2003; Bowman et al., 2005; Bello et al., 2014; Jiang et al., 2016; Yin et al., 2016; Bello et al., 2019), there are no reports of effects on this section of the avian excurrent duct system. The

excurrent duct system of birds comprises the epididymal region and ductus deferens (Maruch et al., 1998). The milieu in these ducts as a result of secretory and absorptive functions enhance the fertilizing capacity of sperm (Jones and Murdoch, 1996). The epithelia of the epididymal region ducts express androgen/estrogen receptors are involved in the regulation of the functions of these reproductive ducts (Goyal et al., 1997; Hess et al., 1997; Kwon et al., 1997; Hess et al., 2001; Oliveira et al., 2004, 2011). These tissues may be sensitive to endocrine-disrupting chemicals including DBP. The present study, therefore, was designed to investigate the histological and ultrastructural changes in the epithelia of the rete testis, efferent ductules and epididymal duct of the mature male Japanese quail, following treatment during the pre-pubertal period with various doses of the DBP for 30 days.

2. Materials and methods

2.1. Chemicals

Di-*n*-butyl phthalate (DBP) (CAS Number 84−74-2—technical grade-99 % purity) was purchased from Sigma-Aldrich (Pty) Ltd (Johannesburg, South Africa).

2.2. Animals and management

Pre-sexed male Japanese quail (*n* = 25; *Coturnix japonica*) were obtained from the Aviary Unit, Irene Animal Improvement Research Station, Pretoria. At the time of hatching, the temperatures were maintained at 35–37 °C and then slowly decreased by 0.5 °C/day until there was a temperature of 16–23 °C at 4 weeks of age (pre-pubertal stage). During the experimental period, the quail were housed in an area where there was imposing of a controlled photoperiodic regimen (16 h light: 8 h dark) and temperature (25 ± 2 °C) with a relative humidity of 50 ± 5% in battery cages (46 × 95 × 51 cm).The birds were housed at the Poultry Research Unit of the Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, South Africa. The quail were individually identified, using wing-tags, and were fed a standard commercial high protein diet for poultry (Obaro Feeds®, Pretoria, South Africa), with free access to fresh water. All procedures were conducted according to the South African National Standard guidelines for the Care and Use of Laboratory and Research Animals (SANS, 2008). The study was approved by the University of Pretoria's Animal Ethics Committee (approval number A065−12).

2.3. Experimental design and dosing regimen

The experiment was conducted using procedures consistent with avian toxicity testing as stipulated by the Organization for Economic Co-operation and Development (OECD, 2010). At 4 weeks of age (pre-pubertal), the birds were randomly assigned to five dosage groups with five birds per group. Birds of Group 1 (control) were administered a corn-oil vehicle (a dose of 1 ml/kg per day), while the birds assigned to Groups 2, 3, 4 and 5 (treatment) were administered via the intra-gastric route daily for 30 days,10, 50, 200 and 400 mg of DBP/kg body weight dissolved in corn oil, respectively. Puberty was determined as the first day of release of cloacal foam (Sezer et al., 2006). Cloacal gland foam production was assessed in

the birds of the control and DBP-treated groups between 6 and 7 weeks post-hatching, by gently squeezing the foam gland. The group administered the smallest (10 mg/kg/d) dose was selected based on the No-Observed-Adverse-Effect-Level (NOAEL) as determined in previous studies in Japanese quail (Bello et al., 2014, 2019).

2.4. Behavioural and clinical assessment

During the experimental period, the birds were weighed once a week, using a precision digital laboratory balance (MII-300 UWE digital precision weighing balance, Algen Scale corporation® Bohemia, NY) and the dose of DBP was adjusted accordingly. The birds were further observed daily for any behavioural changes or clinical symptoms of disease.

2.5. Sample collection

After the 30-day experimental period, the birds were weighed and euthanized using carbon dioxide (CO_2) inhalation. There was an incision made into the thoraco-abdominal cavity and the intestinal tract to expose the testes with epididymal regions. The testicular (left and right) weights, for both the control and DBP-treated groups, were recorded using a precision digital laboratory balance (MII-300 UWE digital precision weighing balance, Algen Scale corporation® Bohemia, NY). Tissue samples (~1 mm^3 blocks) were collected from both the left and right epididymal region (comprising the rete testis, efferent ductules and epididymal duct) and were immediately fixed by immersion in 4% glutaraldehyde in 0.13 M Milliong's phosphate buffer (pH 7.4), for at least 24 h.

2.6. Microscopy

Tissue samples were post-fixed in 1% osmium tetroxide for 2 h, thereafter rinsed in 0.1 M Millonig's buffer, dehydrated in a 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. For light microscopy, semi-thin sections (1 µm thick) were cut and stained with toluidine blue (Bozzola and Russell, 1999). Stained sections were viewed and photographed using an Olympus BX-63 microscope attached to the computer. For transmission electron microscopy (TEM), ultra-thin sections (50−90 nm thick) were cut and stained with lead acetate and counterstained with uranyl citrate. The sections were viewed with a Phillips CM10 transmission electron microscope (FEI, The Netherlands), fitted with an Olympus Megaview III imaging system.

2.7. Histometric measurements of the epithelial height

At 40x magnification under light microscopy, measurements of epithelial heights (in µm) of the different ducts of the epididymal region were made interactively with use of a digitation mouse, on at least five regions selected randomly from three birds in each group for toluidine blue staining. Epithelial height was measured for each group by using an image analyser (CellSens dimension software) connected to Olympus BX-63 microscope. The epithelial height was determined as the linear length from the periductal layer (basal lamina) to the luminal edge.

2.8. Statistical analysis

The histometric data of the epithelial layer height from the birds of the control and DBP-treated groups were tested for normality and homogeneity of variances and then analyzed using One-way analysis of variance (ANOVA) (SPSS 23 software). There were considered to be mean differences with there was a $P \leq 0.05$.

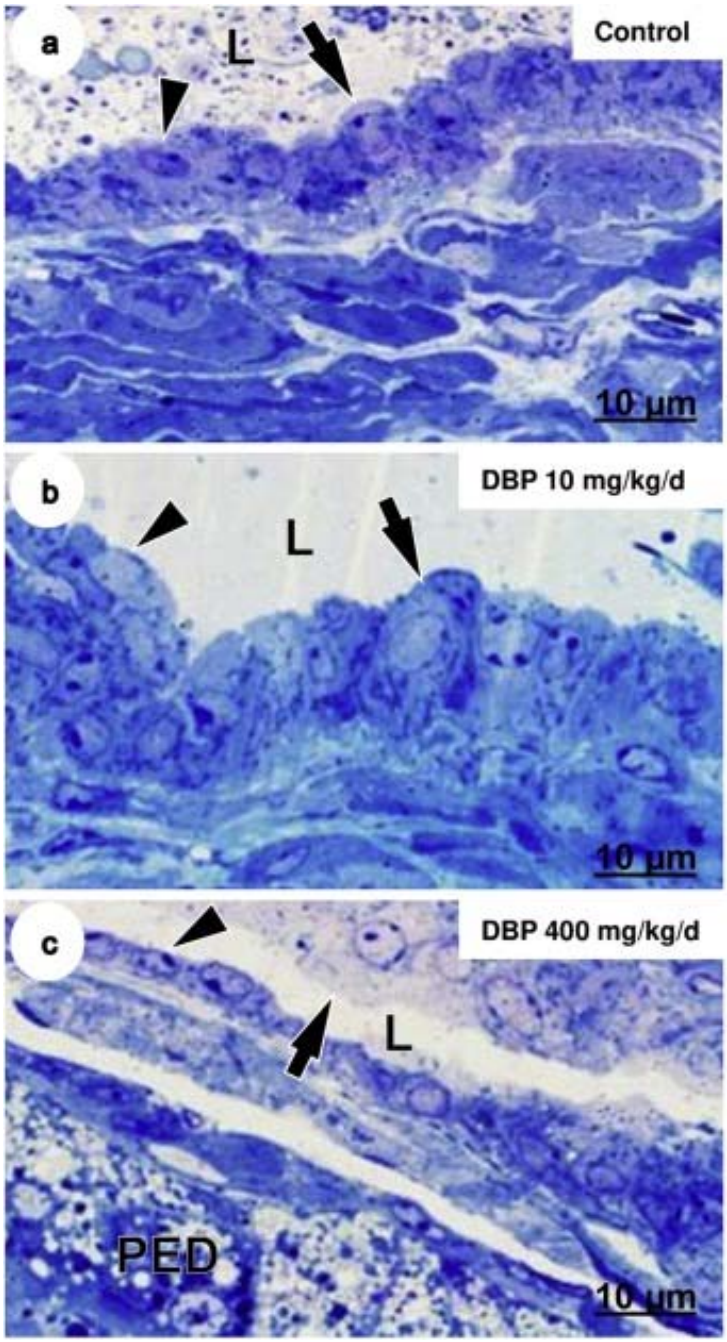

Fig. 1. Light micrographs of the rete testis (RT) epithelium of Japanese quail – control; DEP - (a) 10 (b) and 400 (c) mg/kg/d; (a–c) Squamous (arrowheads) and cuboidal (arrows) cells lining the epithelium of RT; PED, Proximal efferent ductules; L, lumen; Toluidine blue stain

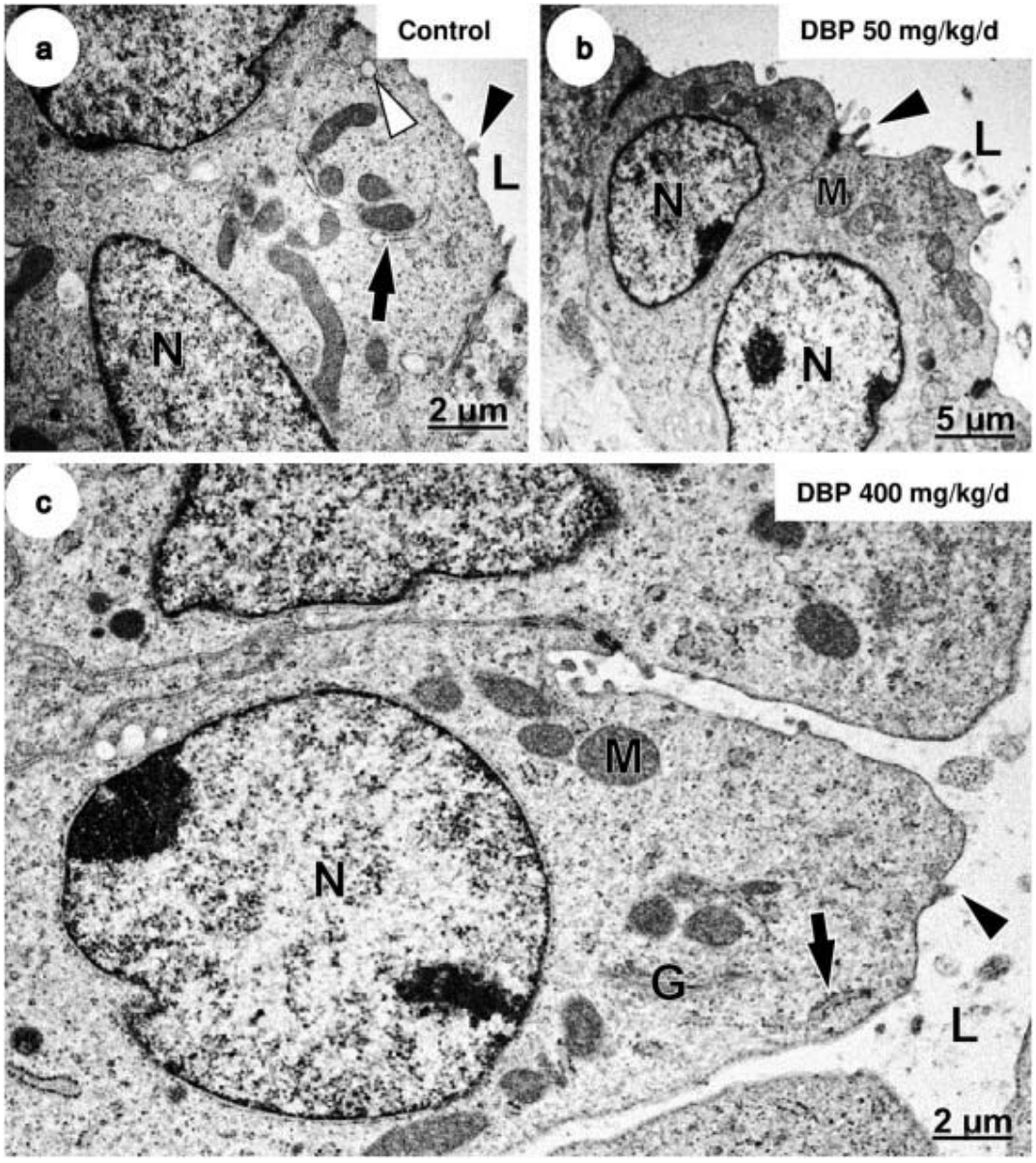

Fig. 2. Transmission electron micrograph of the rete testis epithelium of Japanese quail – control; DEP - (a) 50 (b) and 400 (c) mg/kg/d; Short microvilli (black arrowheads) in the apical surface of the cells lining the RT; Secretory vesicles (white arrowheads), rough endoplasmic reticulum cisternae (arrows), Golgi apparatus (G) and mitochondria (M) are observed in the RT cells; N, Nucleus.

3. Results

3.1. Behavioural and clinical outcomes

There were no obvious clinical symptoms and no mortality in the birds of the control or DBP-treated birds throughout the experimental period.

3.2. Effect of DBP on the epithelium of the rete testis

In both birds of the control and DBP-treated groups, the epithelium of the rete testis was lined by simple squamous to cuboidal cells, which have a few short microvilli on the luminal surfaces (Fig. 1a–c). The cytoplasm of rete testis cells contained organelles with the following characteristics: rough endoplasmic reticulum, Golgi complex, small and elongated mitochondria and a few apical secretory vesicles (Fig. 2a–c). In birds of all groups treated with DBP, there were no obvious histopathological and ultrastructural changes in cells lining the rete testis compared to those of the control group (Figs. 1a–c and 2 a–c).

3.3. Efferent ductules

The proximal and distal segments of the efferent ductules of the Japanese quail were lined with simple columnar or pseudostratified epithelium composed of two cell types: ciliated and non-ciliated. The ciliated cells in control birds were more numerous in the distal segment than in the proximal segment of the efferent ductules (Figs. 3a and 5 a).

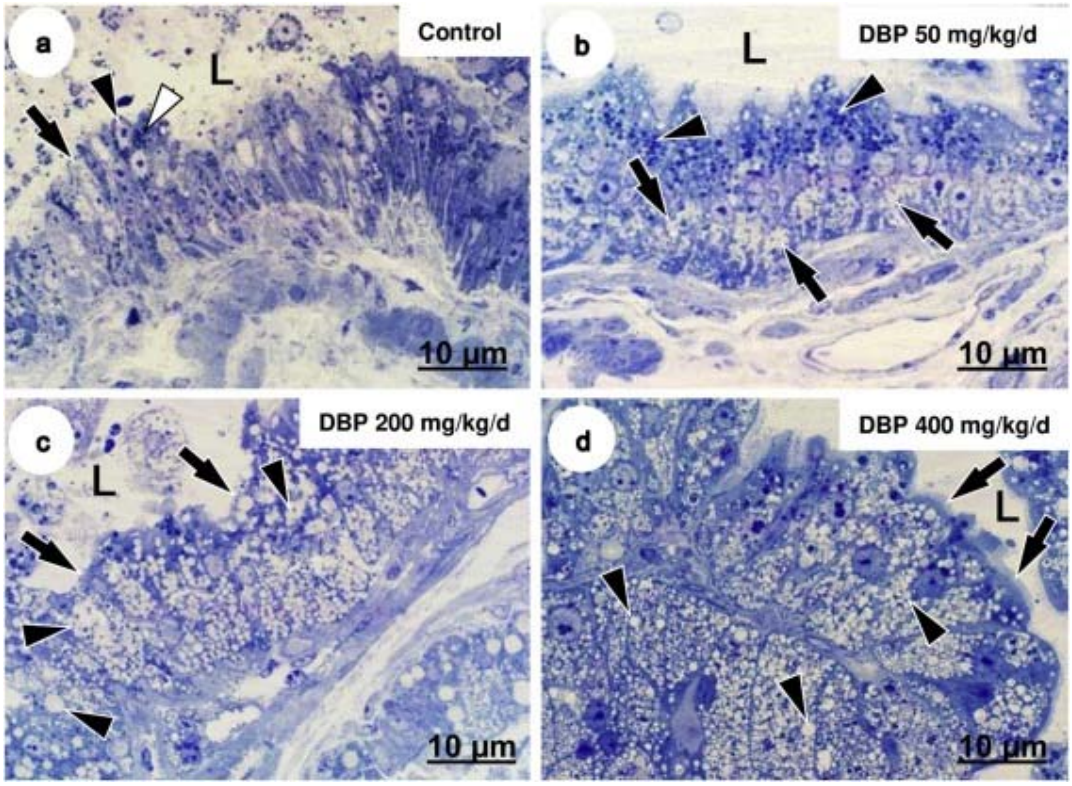

Fig. 3. Light micrographs of proximal efferent ductule (PED) epithelium of Japanese quail - control DEP (a) 50 (b), 200 (c) and 400 (d) mg/kg/d; (a) Unaffected ciliated (arrow) and non-ciliated (black arrowhead) cells in the PED epithelium of control birds; White arrowhead indicate subapical secretory granules in the non-ciliated cell (b) Numerous vacuoles (arrows) in the infra-nuclear region of the PED epithelium; Arrowheads indicate subapical secretory granules in the non-ciliated cell; (c & d) Loss of the normal architecture of the epithelium; loss of the apical cilia and microvilli (arrows); Numerous small and large vacuoles (arrowheads) are observed throughout cytoplasm of the PED epithelial cells; L, lumen;Toluidine blue stain

3.3.1. Effects of DBP on the epithelium of the proximal efferent duct

There were no obvious histopathological and ultrastructural abnormalities in the ciliated and non-ciliated cells of the proximal efferent ductule in birds of the group treated with 10 mg DBP/kg/d compared to those of the control group. In the birds of the group treated with 50 mg DBP/kg/d, the cells lining the proximal efferent ductule had numerous small vacuoles confined to the infra-nuclear cytoplasm (Fig. 3b). In addition, there were noticeable ultrastructural abnormalities in both ciliated and non-ciliated cells such as an increase in the intercellular space, cytoplasmic vacuoles containing dense material, lysosomes and lipid droplets (Fig. 4b). In the birds treated with 200 and 400 mg DBP/kg/d, however, there was a loss of the normal architecture of the epithelium, loss of the cilia and microvilli, as well as the presence of numerous small and large vacuoles throughout the cytoplasm in the proximal efferent ductular epithelia (Fig. 3c, d). In addition, ultra-structurally, degenerative changes in similar areas of this region were characterized by numerous vacuoles, lysosomes, disintegrating cytoplasmic organelles, apoptotic bodies and autophagosomes (Fig. 4c, d).

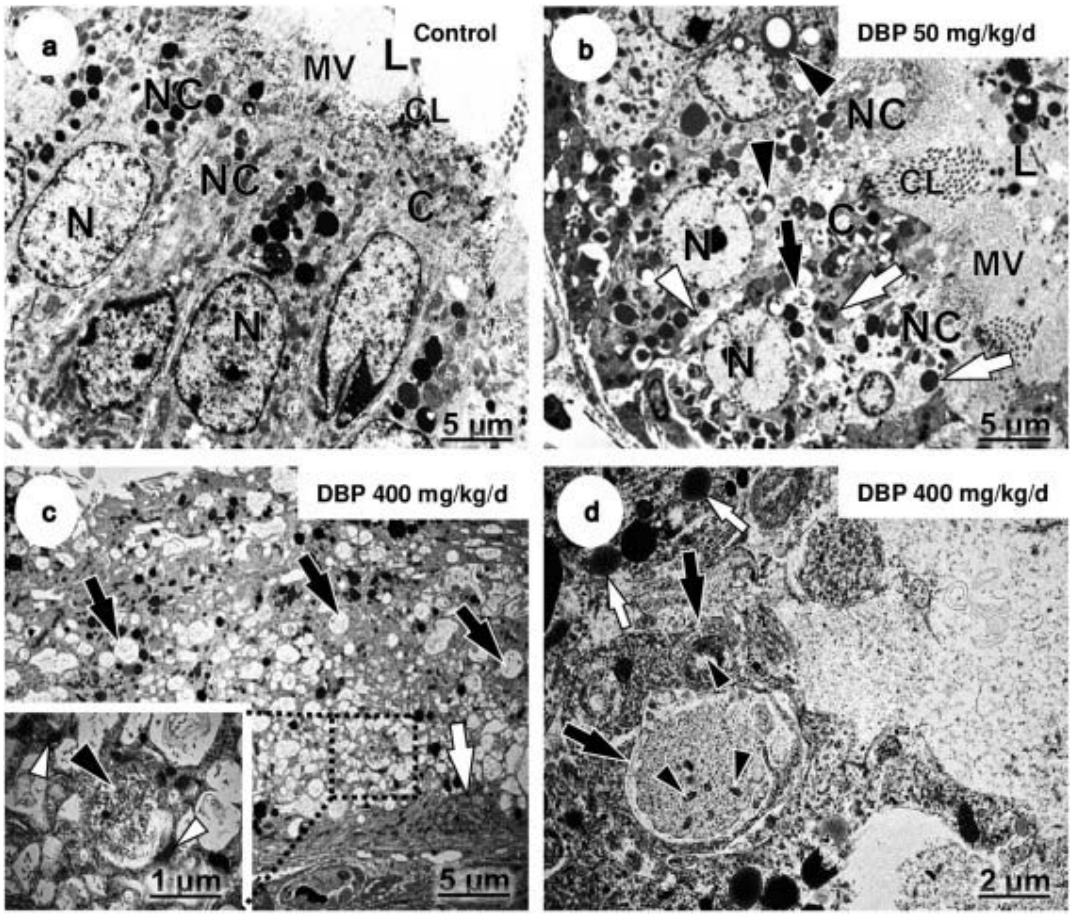

Fig. 4. Transmission electron micrographs of the proximal efferent ductule (PED) epithelium of Japanese quail - control; DEP - (a), 50 (b) and 400 mg/kg/d (c & d) groups; (a) Normal ciliated (C) and non-ciliated (NC) cells in the epithelium of PED; (b) Cytoplasm of C and NC cells in the PED epithelium contains vacuoles enclosing dense material (black arrow), lysosomes (white arrows) and lipid droplets (black arrowheads); White arrowhead indicates an increase in the intercellular space of the PED epithelium (c) Epithelium of the PED

displaying numerous cytoplasmic vacuoles (black arrows) and apoptotic body (white arrow); Inset: Depicts disintegrating nucleus (black arrowhead) and mitochondria (white arrowheads); (d) Autophagosomes (black arrows) containing intracellular components (arrowheads) in the epithelial cells of PED; White arrows indicate lysosomes in the autophagic cells of the PED epithelium; CL, Cilium. L, lumen. MV, Microvillus; N, Nucleus.

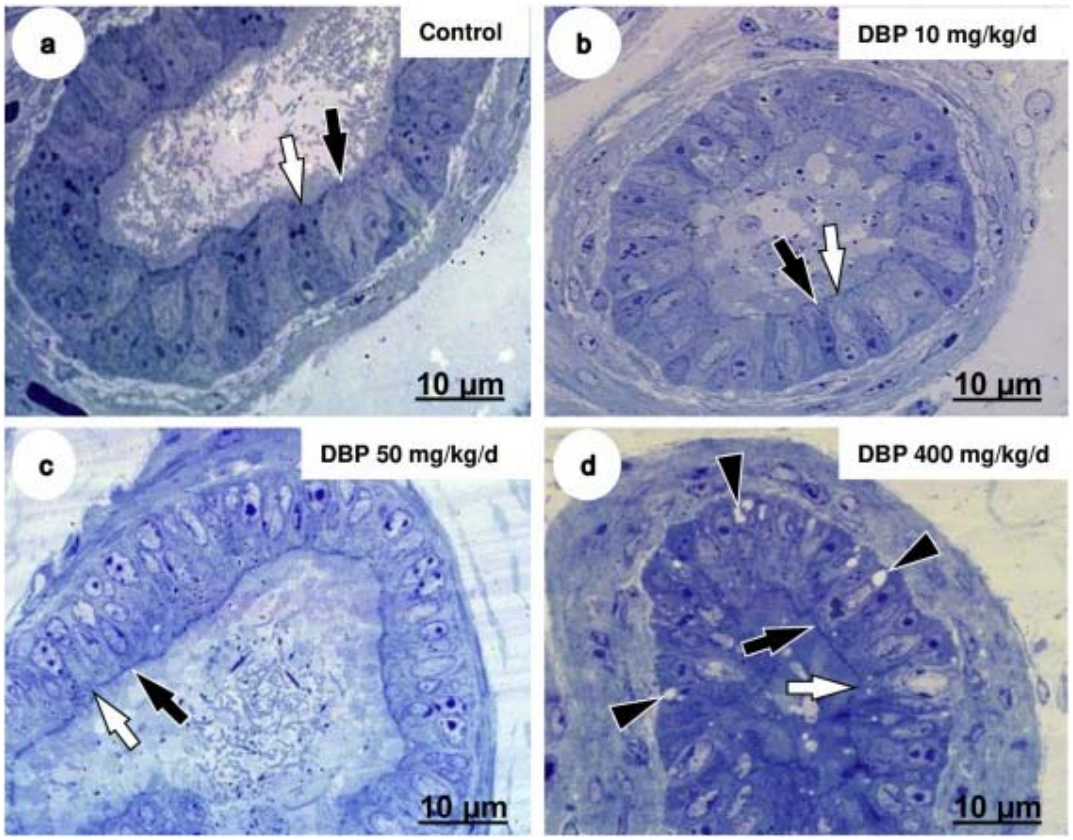

Fig. 5. Light micrographs of the distal efferent ductule (DED) epithelium of Japanese quail – control; DEP - (a), 10 (b), 50 (c) and 400 (d) mg/kg/d; (a–d) Ciliated (black arrows) and non-ciliated (white arrows) cells in the epithelium of DED; (d) Arrowheads indicate vacuoles in the cytoplasm of ciliated cells lining the DED epithelium. Toluidine blue stain

3.3.2. Effects of DBP on the epithelium of the distal efferent ductule

In the birds of the groups treated with 10 and 50 mg DBP/kg/d, there were no obvious histopathological and ultrastructural abnormalities in both ciliated and non-ciliated cells of the epithelial lining of the distal efferent ductule compared to the control group (Figs. 5a–c and 6 a, b). In birds of the groups treated with 200 and 400 mg DBP/kg/d, however, a few vacuoles were visible in the infra-nuclear region of the ciliated cells of the distal efferent ductule (Fig. 5d). In addition, ultrastructural changes such as multi-nucleated ciliated cells and irregular-shaped nuclei in the ciliated and non-ciliated cells were observed in the distal efferent ductular epithelium (Fig. 6c, d).

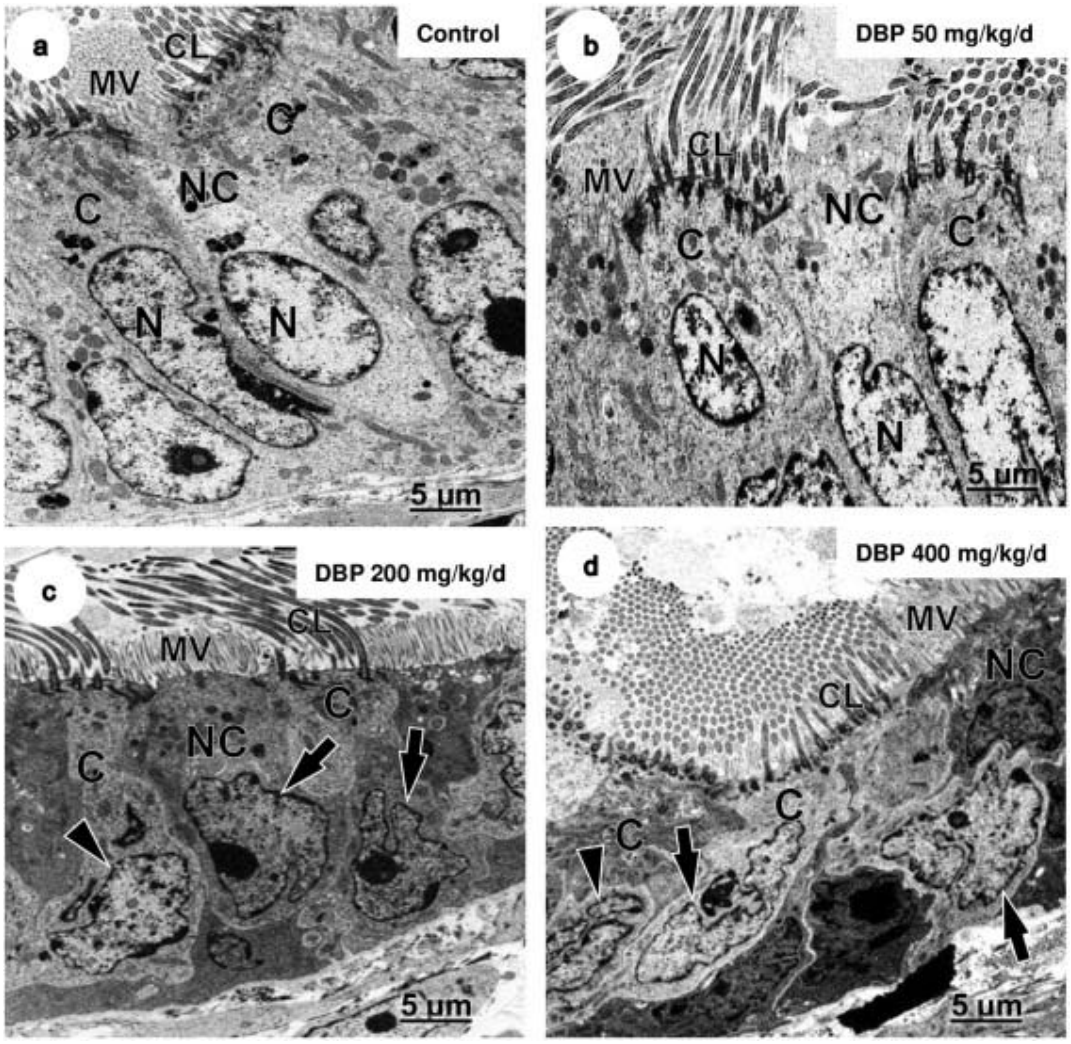

Fig. 6. Transmission electron micrographs of the distal efferent ductule (DED) epithelium in Japanese quail – control; DEP - (a) 50 (b), 200 (c) and 400 (d) mg/kg/d; (a–d) Ciliated (C) and non-ciliated (NC) cells in the DED epithelium; (c & d) Arrows indicate and irregular-shaped nuclei in the C and NC cells of the DED epithelium; Arrowheads depict multinucleated ciliated cells lining the DED epithelium; CL, Cilium; MV, Microvillus; N, Nucleus.

3.4. Effects of DBP on the epididymal duct epithelium

The epididymal duct was lined by simple columnar epithelium, which appeared pseudostratified if cut at an angle and was composed of non-ciliated and basal cells (Fig. 7a). There were no obvious histopathological or ultrastructural changes in the cells lining the epididymal duct in the birds treated with 10 mg DBP/kg/d compared to those of the control group (Fig. 7b and 8 b). Large vacuoles were observed in the supranuclear and infra-nuclear cytoplasm in the epithelium of this region of the birds treated with 50, 200 and 400 mg DBP/kg/d. In addition, the lumen of the epididymal duct was oligospermic in the birds treated with 200 and 400 mg DBP/kg/d (Figs. 7c, d and 8 c, d) compared with those in the control birds, which contained numerous spermatozoa.

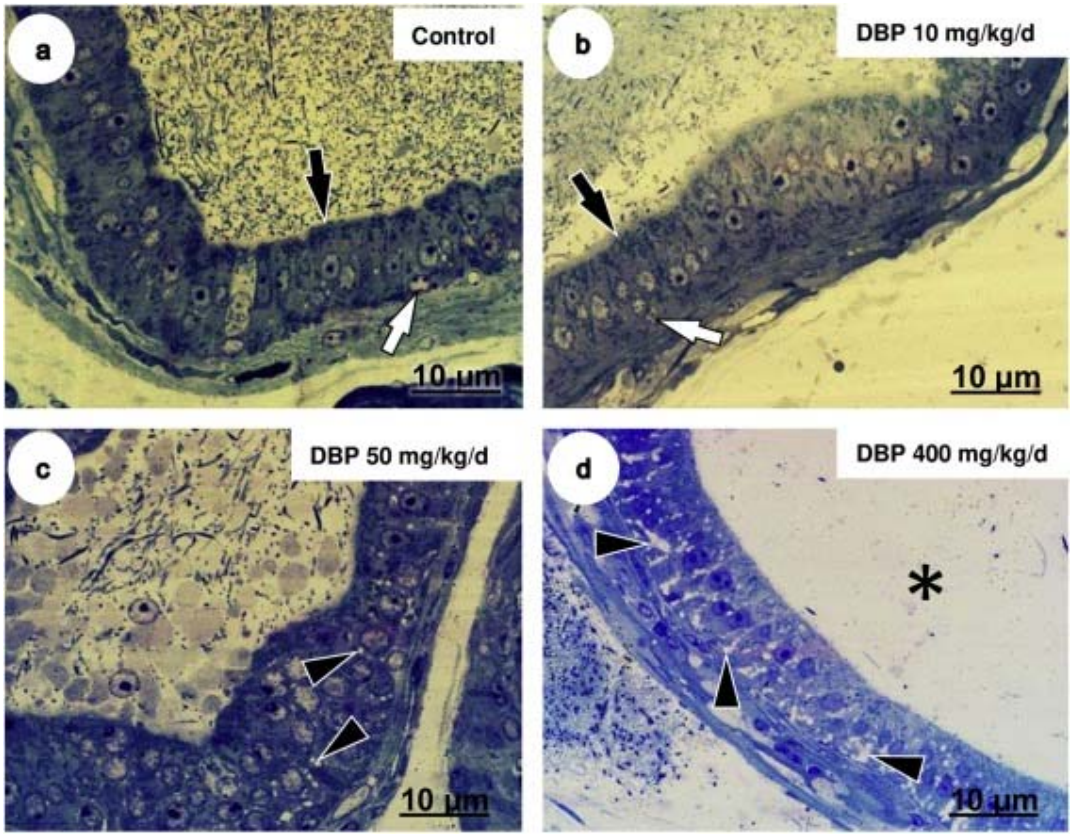

Fig. 7. Light photomicrographs of the epididymal duct (ED) epithelium of Japanese quail – control; DBP - (a), 10(b), 50(c) and 400 (d) mg/kg/d; (a & b) Non-ciliated (black arrows) and basal (white arrows) cells in the epithelia of ED; (c & d) Arrowheads indicate several cytoplasmic vacuoles in the cells of the ED epithelium; (d) Lumen of the ED is oligozoospermic (asterisk); Toluidine blue stain

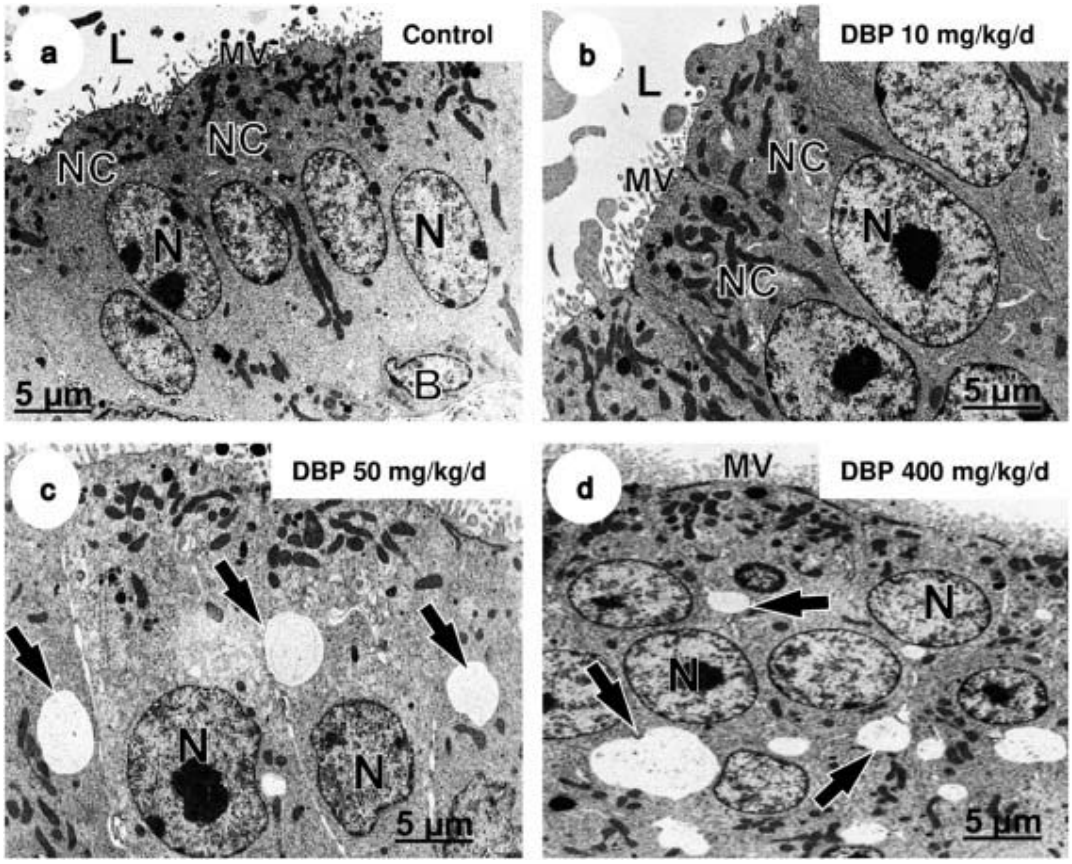

Fig. 8. Transmission electron micrographs of the epididymal duct (ED) epithelium of Japanese quail – control; DBP - (a), 10 (b) 50 (c) and 400 (d) mg/kg/d; (a & b) Unaffected non-ciliated (NC) and basal (B) cells in the epithelium of the ED; (c) Depict large vacuoles (arrows) in the supranuclear cytoplasm of the ED epithelial cells; (d) Arrows indicate large vacuoles that are located more in the infra-nuclear cytoplasm of the ED epithelium; L, lumen; MV, Microvillus; N, Nucleus.

Table 1. The histometrical measurements (MEANS ± SD) of epithelial layer height in the epididymal region of the adult male Japanese quail following treatment with di-*n*-butyl phthalate (DBP) at doses 10, 50, 200 and 400 mg/kg/d prepuberty.

Parameters	Experimental Groups ($n = 3$)				
	Control	DBP (10 mg)	DBP (50 mg)	DBP (200 mg)	DBP (400 mg)
RT (µm)	10.2 ± 2.3^{a}	10.6 ± 1.6^{a}	5.3 ± 1.2^{b}	6.4 ± 0.7^{b}	6.5 ± 0.9^{b}
PED (µm)	43.4 ± 10.5^{a}	33.8 ± 4.7^{b}	34.9 ± 5.4^{b}	30.0 ± 4.3^{b}	33.1 ± 7.9^{b}
DED (µm)	17.3 ± 4.1^{a}	17.1 ± 4.2^{a}	13.4 ± 2.5^{b}	13.8 ± 1.4^{b}	13.9 ± 3.0^{b}
ED (µm)	27.7 ± 3.5^{a}	24.1 ± 2.8^{a}	23.5 ± 4.2^{a}	24.6 ± 3.6^{a}	23.8 ± 3.2^{a}

^{abc}Denotes differences between treatments ($P \leq 0.05$).

RT = Rete testis; PED = Proximal efferent ductule; DED = Distal efferent ductule; ED = epididymal duct.

3.5. Histometric (epithelial height) characteristics

There was no difference in the epithelial height of the rete testis between birds treated with 10 mg DBP/kg/d and control groups. In the birds treated with 50, 200 and 400 mg DBP/kg/d, however, the height of the rete testis epithelium was less ($P \leq 0.001$) than in the control group (Table 1).

The epithelial heights of the proximal efferent ducts in birds of all DBP-treated groups were less ($P \leq 0.001$) compared to the birds of the control group (Table 1). The epithelial height of distal efferent ducts in the birds of the 10 mg DBP/kg/d group did not differ from the birds of the control group, while the epithelial heights in the birds treated with 50, 200 and 400 mg DBP/kg/d were less ($P \leq 0.001$) compared to the control group (Table 1). There was no difference in the epithelial heights in the epididymal duct between birds of all DBP-treated and the control group (Table 1).

4. Discussion

The objective of this experiment was to investigate the effects of various doses of DBP on the histological and ultrastructural features of the epithelial layers of the ducts comprising the epididymal region of post-pubertal, male Japanese quail (8 week post-hatching) when dosed for 30 days commencing at the pre-pubertal stage (4 week old). The avian epididymal region has important functions in the maturation and viability of spermatozoa (Clulow and Jones, 2004). Any alterations in these ducts, therefore, may lead to infertility. The results of the current study indicated that DBP induces several histopathological and ultrastructural changes in the epithelial layers of the epididymal region ducts. The lesions in the birds of the groups treated with 200 and 400 mg DBP/kg/d were more pronounced than in the birds treated with 50 mg DBP/kg/d, while there were no obvious structural changes in the birds treated with 10 mg DBP/kg/d when compared with control birds. Results from previous studies indicate that DBP induces dose-dependent toxicity in the male reproductive organs in both experimental mammals and birds (Zhou et al., 2010, 2011; Bello et al., 2014; Sahin et al., 2014; Bello et al., 2019).

4.1. Effect of the DBP on the epithelium of the rete testis

Results of the current study also indicated there were no histopathological and ultrastructural abnormalities induced in the cells lining the rete testis in birds of all groups treated with DBP. The results from the histometrical analysis, however, indicated there was a lesser epithelial height of the rete testis in the birds treated with 50, 200 and 400 mg DBP/kg/d. Rivas et al. (2003) attributed the lesser epithelial height of the rete testis to a disruption in androgen/estrogen balance. Hess (2018b) and Lee et al. (2000), however, reported there was a lesser epithelial height of the rete testis that might be due to the inhibition of fluid reabsorption in efferent ductules that results in accumulation of testicular fluid in this region with subsequent pressure atrophy. It is surmised that the decrease in the epithelial height in the rete testis in the present study could also be due to the accumulation of fluid in the lumen of this region ascribed to the inhibition caused by DBP on the capacity of the ducts to absorb fluid from the lumen.

4.2. Effect of DBP on the efferent ductule epithelium

In the present study, the birds treated with 200 and 400 mg DBP/kg/d had more morphological abnormalities in the epithelia of proximal and distal efferent ductules when compared with the control birds. There were lesser epithelial heights in both ducts while there were primary cytological abnormalities in the epithelium of the proximal efferent duct. There were degenerative changes such as numerous cytoplasmic vacuoles, lysosomes and disintegrating cytoplasmic organelles in the epithelium of the proximal efferent ductule. The efferent ductules are a primary site of reabsorbing most of the testicular fluid (Bahr et al., 2006), which is regulated by direct physiological control and steroid hormone receptor activity (Hess, 2018a). In addition, estrogen binding to the alpha receptor has are essential for maintaining the normal morphology of efferent ducts (Nanjappa et al., 2016). There is estrogenic activity of DBP (Chen et al., 2014), and steroidogenic enzymatic pathways are altered in function in the testis of the Japanese quail when there are treatments with DBP (Bello et al., 2014). It, therefore, is possible that the morphological changes in the epithelial cells of the proximal efferent ductule in the present study are associated with alterations in the testicular steroidogenic enzyme pathways or a direct effect of the DBP through activation of estrogen receptor alpha in this tissue.

Furthermore, there were apoptotic bodies and autophagosomes (Fig. 4c, d) in the epithelium of the proximal efferent ductule in the birds treated with 200 and 400 mg DBP/kg/d. Apoptosis and autophagy were also noticeable in goat (Zhang et al., 2017) and rat (Duan et al., 2017) Sertoli cells and rat ovarian granulosa cells (Liu et al., 2020) after treatments with endocrine disruptors. Results from previous studies in mouse ameloblast cell tissue culture (Suzuki et al., 2015) and goat testis Sertoli cells (Zhang et al., 2017) indicated apoptosis or autophagy can be induced by oxidative stress. Di-*n*-butyl phthalate induced oxidative stress in testicular and epididymal tissues of post-pubertal Sprague-Dawley rats (Zhou et al., 2010, 2011). Consequently, apoptosis and autophagy in the epithelium of the proximal efferent duct in the present study might be due to the stimulation of the oxidative mechanisms in the epithelial cells by DBP or its metabolites.

4.3. Effect of DBP on the epididymal duct epithelium

The epididymal duct of birds is a highly convoluted network of tubules, which has an essential function in the maintenance of viable sperm by providing an optimal milieu for the micro-environment that is needed to complete sperm maturation (Clulow and Jones, 1982). For maintenance of structure and functions, the epididymis is androgen-dependent (Jiang et al., 2016). In the present study, there were large cytoplasmic vacuoles (Fig. 8c, d) in the epithelium of the epididymal duct of the birds treated with 50, 200 and 400 mg BBP/kg/d. There are anti-androgenic effects of DBP, therefore, the cytoplasmic vacuoles in the epididymal duct epithelium observed in the present study might have been due to the anti-androgenic activity of this compound (Borch et al., 2006).

The oligospermia observed in the birds treated with DBP 200 and 400 mg/kg/d in the present study when compared with non-treated quail and those treated with 10 and 50 mg/kg/d DBP is consistent with findings in post-pubertal Sprague-Dawley and Wistar rats following treatment with DBP (Zhou et al., 2011; Patel et al., 2014). Results of previous

studies on the testes of the Japanese quail (Bello et al., 2014) and Sprague-Dawley rats (Zhou et al., 2010), indicate DBP treatment induces an incremental reduction in the number of spermatids in the seminiferous tubules. Furthermore, Bello et al. (2019) reported that DBP alters Sertoli cell morphology, which could result in spermatogenic failure. The lesser number of spermatozoa stored in the lumen of the epididymal duct in the present study might have been associated with DBP-induced testicular toxicity.

5. Conclusion

The results of the current study confirmed that treatment of quail with DBP at doses of 10, 50, 200 and 400 mg/kg/d, commencing pre-puberty, induced dose-dependent morphological changes in the various epithelia of the epididymal region of the post-pubertal male Japanese quail. There were marked histopathological changes induced by DBP in the epithelium of the proximal efferent ductule, indicating that this segment is extremely sensitive to the effects of DBP, a known endocrine disrupting chemical. In addition, the lumen of the epididymal duct was oligospermic. Overall, the changes in the epididymal ducts of the Japanese quail treated with DBP could cause infertility.

Declaration of Competing Interest

The authors report no declarations of interest.

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