ACADEMIA JOURNAL OF BIOLOGY 2024, 46(4): 57–70
DOI: 10.15625/2615-9023/21119

INVESTIGATION OF THE EFFECTS OF DIISOBUTYL PHTHALATE ON RAT TESTICULAR TISSUE: A HISTOPATHOLOGICAL AND MORPHOMETRIC EVALUATION

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Received 15 July 2024; accepted 9 December 2024

ABSTRACT

Phthalates are a group of chemicals used to make plastics more durable, and they are often called plasticisers. Additionally, these chemicals are found in hundreds of products such as floor coverings, lubricating oils, and personal care products (soaps, shampoos, hair sprays). Consumer products containing phthalates can result in human exposure through direct contact and use, indirectly through leaching into the other products or general environmental contamination. In this study, the effects of Diisobutyl phthalate a commonly used phthalate, were investigated histopathologically and morphometrically to determine whether it is one of the causes of increased infertility in recent years. Two study groups of albino *Wistar albino* rats (total n: 40) were formed; the control group (untreated control group, solvent-corn oil the control group) and the experimental group. DiBP was administered by oral gavage to the experimental group in 3 different doses (0.25–0.5–1 mL/kg/day) mixed with corn oil every day for 28 days. At the end of the experiment, testicular tissue samples taken from all the experimental and control animals were evaluated histopathologically and morphometrically by light microscopy after routine preparation. Degeneration/atrophic tubules were quite prominent in the sections. Tubules containing degenerated germ cells and tubules devoid of germ cells were observed. It was determined that in most tubules, only tubules covered with Sertoli cells remained due to germ cell death. In addition, multinucleated giant cells were frequently encountered in such tubules. Dilatation and thickening in the basal lamina of the seminiferous tubule were accompanied by decreased PAS-positive reaction. The morphometric results supported the histopathological findings. Significant dose-related morphometrical changes ($p<0.0001$), including seminiferous tubule diameter, tubular lumen diameter, spermatogenic cell line height and basal lamina thickness were observed between the control and administration groups. According to the control, sham and G1, the number of these multinucleated cells (MGC) increased in G2 and G3 but these increases were statistically insignificant ($p > 0.9999$). In conclusion, it was observed that irreversible damage occurred in the testicular tissues of DiBP-exposed groups, and it was decided that this could be the cause of infertility. Therefore, we recommend the use of an alternative plasticiser with proven reliability.

Keywords: Diisobutyl phthalate, testis, infertility, histopathology, histomorphometry.

Citation: Yücel Başımoğlu Koca, 2024. Investigation of the effects of diisobutyl phthalate on rat testicular tissue: a histopathological and morphometric evaluation. *Academia Journal of Biology*, 46(4): 57–70. https://doi.org/10.15625/2615-9023/21119

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INTRODUCTION

Spermatogenesis is a complex process that involves interactions between supporting somatic cells (Sertoli cells) and germ cells within the seminiferous tubules, and it plays a crucial role in male reproduction. Toxic substances can negatively impact male reproductive function, including the testes. Although molecular studies help understand the effects of toxicants on the reproductive system, it is often possible to determine potential toxicity by detailed examination of early morphological changes in target cells.

Phthalates are extensively used as plasticisers in PVC plastics. Therefore, many consumer products contain specific members of this family of chemicals (ATSDR, 1995, 1997, 2001, 2022; CERHR, 2000, 2003, 2005). Since 1999 the six phthalates, namely diethylhexyl phthalate (DEHP), di-n-butyl phthalate (DBP), butyl benzyl phthalate (BBP), diisononyl phthalate (DiNP), diisodecyl phthalate (DiDP) and di-n-octyl phthalate (DnOP), have been subject to temporary bans due to their recognised carcinogenic and mutagenic effects. DiNP, DiDP and DnOP simultaneously with DBP, DEHP and BBP were recently banned from use in toys that children can put into their mouths, regardless of age category, due to their reproductive toxicity (Borch et al., 2006).

Diisobutyl phthalate (DiBP) is a member of the phthalate ester class of chemicals and is used as a plasticizer to provide flexibility and durability to a wide variety of industrial and consumer products, including paints, lacquers, printing ink, pulp and paper, carpet, concrete, nail polish, and cosmetics (HSDB, 2017). Because of its use in household products, people are exposed to DiBP via food and indoor environments (Wormuth et al., 2006). DiBP is absorbed via oral ingestion (Koch et al., 2012) and dermal exposure (Elsisi et al., 1989), and is rapidly hydrolyzed to its primary metabolite, monoisobutyl phthalate (MIBP). DIBP and MIBP are distributed systemically in the blood (Strucinski et al., 2006), and these chemicals can be transferred to human breast milk (Fromme et al., 2011; Latini et al., 2009) and cross the placental barrier (Wittassek et al., 2009).

Yost et al. (2019) study results support DiBP as a children's health concern and indicate that male reproductive and developmental toxicities are hazards of DiBP exposure, with some evidence for female reproductive, kidney and liver toxicity. Some phthalates, including di-n-butyl phthalate (DBP), diethylhexyl phthalate (DEHP) and butyl benzyl phthalate (BBP), have been identified to disrupt reproductive development in male mice (Barlow et al., 2004). Borch et al. (2006) reported that anogenital distance was statistically reduced on day 20/21 of pregnancy day (GD), along with a decrease in testicular testosterone production and content, following DiBP administration. In the same study, known histopathological effects of DBP and DEHP, such as Leydig cell hyperplasia, Sertoli cell vacuolisation, centrally located gonocytes and multinucleated gonocytes, were observed in the testes of animals exposed to DiBP at GD20/21.

Despite this knowledge, these phthalates are still used in various fields. Diisobutyl phthalate (DiBP) shares a similar structure and application properties with di-n-butyl phthalate (DBP) but it is preferred due to its lower production cost, density and freezing point (The Chemical Company., 2021). Although there are studies investigating the effects of DBP on fetal testes, histopathological information on the adult male reproductive effects of DiBP in experimental animals is limited.

Histopathological evaluation of the testis plays a crucial role in drug safety assessment and evaluation of environmental toxicants. Understanding the potential toxicity of phthalates in rats can provide valuable insights into various male reproductive disorders in humans. Biomonitoring studies show a trend towards increased exposure to diisobutyl phthalate (DIBP) rather than the proven toxic dibutyl phthalate (DBP). With this in mind, this study focused on investigating whether DiBP as an example of a plasticizer could cause infertility in humans.

MATERIALS AND METHODS

Chemicals

Diisobutyl phthalate (DiBP; CAS No: 84-69-5) was purchased from Chemical Service Inc. (West Chester) (Fig. 1). All other chemicals were obtained from Sigma Chemical (St. Louis, MO).

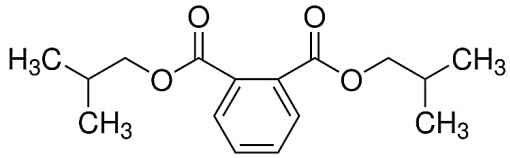

Figure 1. Chemical structure of Diisobutyl phthalate (DiBP) ($C_{16}H_{22}O_4$)

Animals and ethical approval

Male *Wistar albino* rats weighing between 200–250 g were used in this study. A total of 40 rats were obtained from Aydın Adnan Menderes University (ADU)-Faculty of Veterinary. The rats were housed at a temperature of 22 ^{o}C with a 14-hour light and 10-hour dark cycle. They were provided with standard food pellets and water ad libitum. All procedures involving the care and experimentation on the rats were conducted by the guidelines and regulations set by the Local Animal Experimentation Ethics Committee of Aydın Adnan Menderes University (approval number: FEF-13006) and adhered to the Animal Ethics Rules of Aydın Adnan Menderes University. The handling of the rats followed the protocols outlined in the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Experimental design

After 10 days of acclimation, the rats were randomly assigned to control and experimental groups. All groups consisted of eight animals. The control group was further divided into two subgroups: one received no treatment (untreated control), while the other was administered corn oil (solvent control). The experimental groups were orally administered Diisobutyl phthalate (DiBP) dissolved in corn oil at three different doses: 0.25 mL/kg (Group I), 0.5 mL/kg (Group II), and 1 mL/kg (Group III). The dosages of DiBP were determined based on LD50 values (10,400 mg/kg body weight for rats) and the body weight of the rats (European Commission, 2000, 2004). The oral administration of DiBP was carried out daily for 28 days, following the guidelines outlined in the OECD guideline 407. The animals' body weights were recorded weekly, per the protocols set by the Organization for Economic Co-Operation and Development (OECD, 1995).

Histopathological method

At the end of the experiment, rats were euthanised by cervical dislocation. Testes were carefully dissected from each rat and then fixed in neutral buffered formalin (BNF) solution for 24 h at 4 ^{o}C. Subsequently, the tissues were dehydrated using a graded series of ethanol, cleared with xylene, and embedded in paraffin. Sections with a thickness of five micrometres were prepared and stained with Hematoxylin-eosin (HE) to examine the overall morphology of the tissue. Additionally, Periodic Acid Schiff-Hematoxylin (PAS-H) staining was applied to identify neutral glycoconjugates and distinguish the basal lamina of the seminiferous tubule in testicular tissue samples (Bancroft & Cook, 1994). The sections were examined and captured using an Olympus BX51 brightfield microscope equipped with an Olympus E-330 digital camera (Shinjuku City, Japan).

Testicular morphometry measurements

For the morphometric analysis, 40 total sections were taken from each group, with 5 sections obtained from each rat. In each section, measurements were performed on different parameters of 10 seminiferous tubules. To capture the digital photograph of the entire testis, an Olympus BX51 microscope with an Olympus E-330 digital camera from Shinjuku City, Japan was used. The required magnification was selected to obtain clear images. Five random pictures were taken for each histological sample to ensure representative values for each measured parameter in each rat. This

approach allowed the calculation of mean values for the investigated parameters.

The morphometric analysis was conducted by the same author in a blinded manner. Various parameters including seminiferous tubule diameter (STD), tubular lumen diameter (TLD), germ cells/spermatogenic cell line height (SCLH), basal lamina thickness (BLT), and multinucleated giant cells (MGC) were measured and compared with the control group. PC-based image analysis software (Kameram, Micro System Ltd., Istanbul Technical University, Tecnokent ARI-1 building, Argenit Company-Istanbul, Turkey) was used to perform the morphometric measurements on the sections. The measurements were uploaded to the Graph Pad Prism (Ver 9) statistics program and an ANOVA test was performed. A comparison of the changes in the application groups with the control group was made with the Kruskal-Wallis test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Histopathological findings

Typical testicular morphology was observed in the histological examination of the control group. The seminiferous tubules were filled with germ cells arranged closely together and in different stages of meiosis. Various stages of the spermatogenic series were observed, including spermatogonia, spermatocytes, spermatids and spermatozoa, arranged from the outermost to the lumen of the tubules (Figs. 2a–e). Sertoli cells, among the spermatogenic cell line, were identified in the seminiferous tubules with their pale appearance, invaginated nuclei and prominent nucleolus (Figs. 2b, c). Leydig cells were observed close to blood vessels within the connective tissue supporting the seminiferous tubules. Structures containing neutral glycoconjugates appeared in pink-purple in the PAS-treated testicular tissue sections of the control group. In particular, the basal lamina of seminiferous tubules exhibited a strong PAS-H positive reaction (Figs. 2d, e).

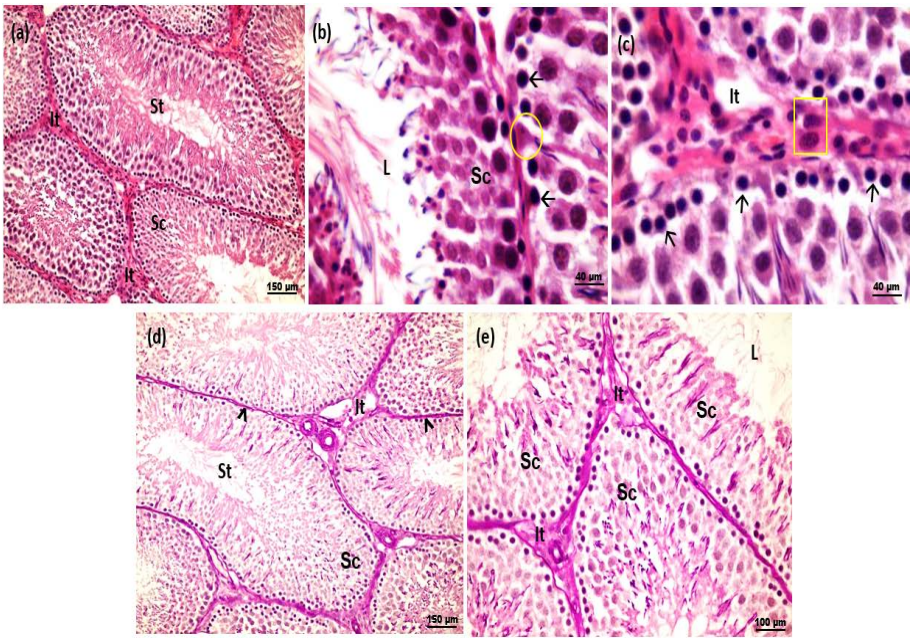

Figure 2. Photomicrographs of the testes of the control group. Seminiferous tubules (St), seminiferous tubule lumen (L), basal lamina of the seminiferous tubule (>) spermatogenic cell line (Sc), spermatogonium (→), Sertoli cell in seminiferous tubules (circle), Leydig cell (square) in interstitial tissue (It). Staining: (a,b,c): Hematoxylin-eosin., (d,e): PAS-H

Along with the notable macroscopic shrinkage in the testes, irreversible histological alterations were observed in the experimental groups. In all the DiBP-treated groups, there was disruption and reduction in the population of spermatogenic cells, leading to arrest in spermatogenesis (Figs. 3–7). This situation was found to increase in a dose-dependent manner. Although the observed pathological changes were similar across all the experimental groups, they appeared more severe in the medium and high-dose groups.

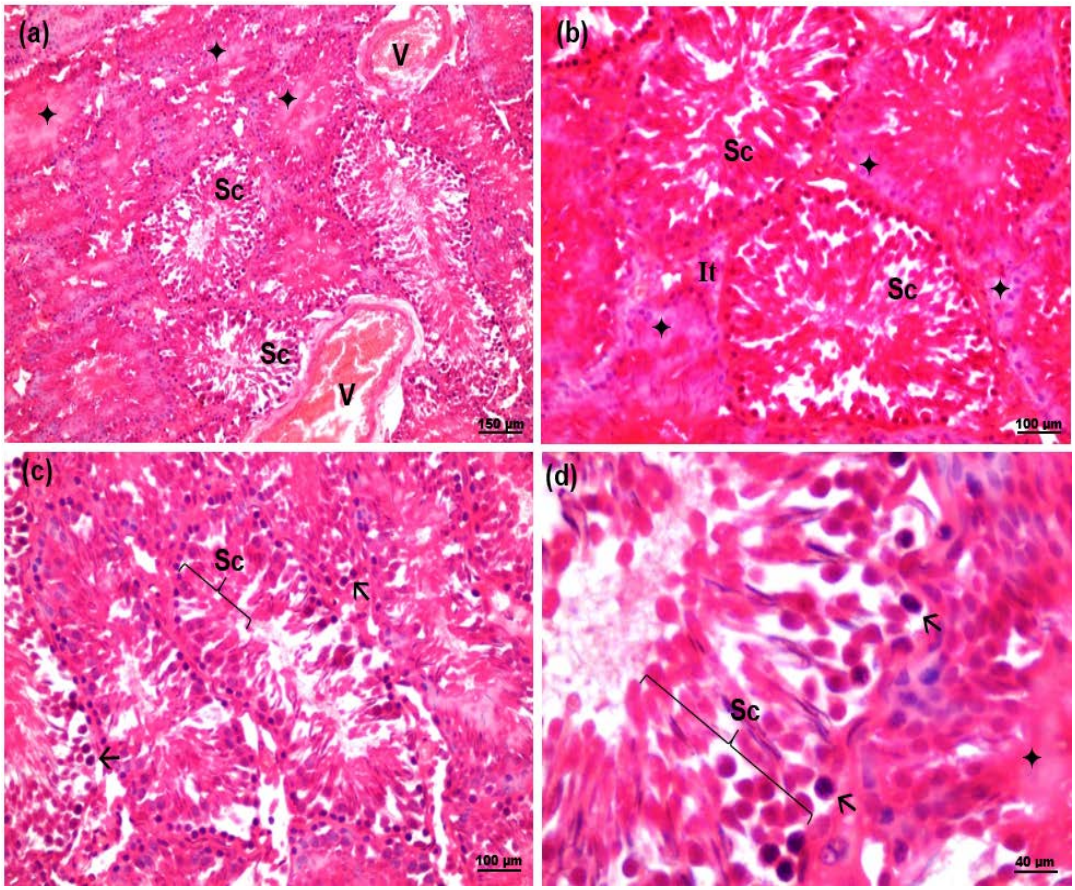

Figure 3. Histological sections of the testes of experimental group-1 (G1-0.25mg/kg/day). Photographs show the staining difference in the seminiferous tubules (✦) and a relatively regular line of spermatogenic cells (Sc). Spermatogonium (→), interstitial tissue (It), blood vessel (V). Staining: H-E

In the experimental group-1 (G1-0.25 mg/kg/day), although the seminiferous tubules appeared relatively filled with spermatogenic cells, a change in staining, particularly in the spermatogonia, was observed (Figs. 3a, b). The application of PAS-H staining revealed dilatation of the basal lamina in most seminiferous tubules (Figs. 4a–d). Additionally, oedematous interstitial tissue was noted (Fig. 3c).

In experimental group 2 (G2-0.5 mg/kg/day), there was a noticeable decrease in the spermatogenic cell line lining the seminiferous tubules, indicating tubular atrophy (Figs. 5a, c). The lumen of the seminiferous tubules appeared filled with loosely and scattered arranged germ cells (Figs. 5b, c). Some tubules contained immature spermatogenic cells within their lumen. Additionally, immature spermatogenic cells were observed in the

interstitial tissue surrounding the tubules with damaged basal lamina (Fig. 6b). The majority of cells present in the tubules of DiBP-treated animals were round spermatids and spermatocytes, contributing to the expanded tubular lumen. The germinal epithelium within the seminiferous tubules appeared degenerated and devoid of sperm. Moreover, empty vacuolar spaces were observed between Sertoli cells in areas of tubular germ cells (Fig. 5d). The sections also indicated the presence of oedema in the interstitial tissue, degeneration of the basal lamina of the seminiferous tubules, and an increase in Leydig cells (Figs. 5b, c, d).

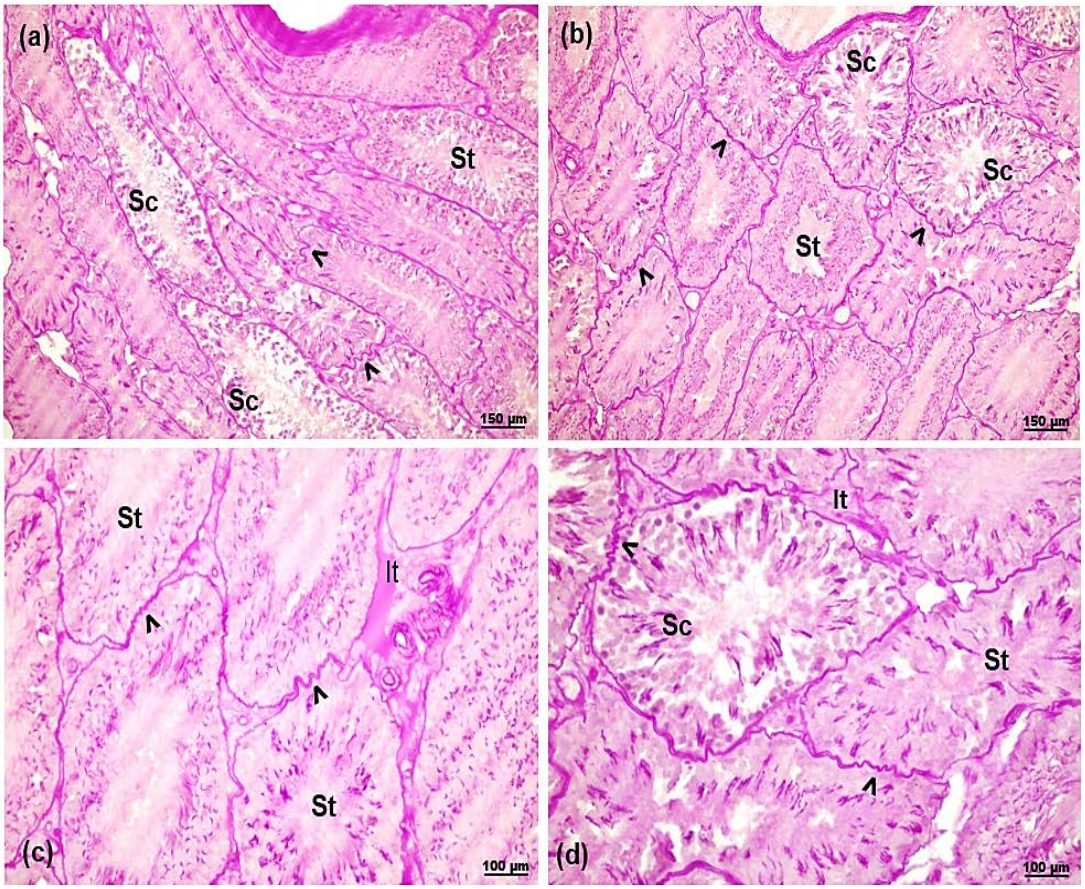

Figure 4. Photomicrograph of seminiferous tubules of experimental group-1 (G1-0.25 mg/kg/day). Advanced dilatation of the basal lamina of the seminiferous tubules (>). Seminiferous tubules (Sc), interstitial tissue (It). Staining: PAS-H

In experimental group 3 (G3-1 mg/kg/day), it was observed that a significant number of germ cells were lost, resulting in tubules predominantly lined with Sertoli cells similar to G2 (Figs. 7a, b). The tubules were almost completely covered by Sertoli cells, indicating a condition known as Sertoli cell-only syndrome (Fig. 7c). In particular, in G2, numerous multinucleated giant cells were frequently observed within the seminiferous tubules (Figs. 6a, c, d). Other notable findings included the presence of oedema in the interstitial tissue, a decrease in PAS-positive staining indicating the presence of neutral glycoconjugates, and degeneration of the basal lamina of the seminiferous tubules. In G3, decreased PAS-positive reaction and dilatation of tubules were accompanied by thickening of the basal lamina (Figs. 7d–f).

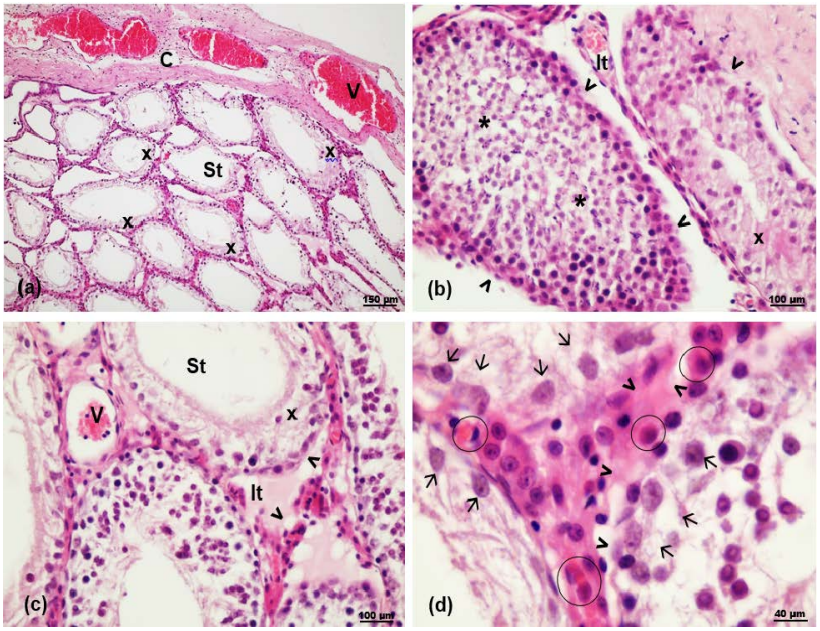

Figure 5. Photomicrographs of the testis of experimental group-2 (G2-0.5 mg/kg/day). a and c; Seminiferous tubules containing few spermatogenic cells (x), b; seminiferous tubule, whose lumen is filled with immature spermatogenic cells, d; degeneration of the basal lamina of the seminiferous tubule (>). Seminiferous tubule (St), Sertoli cells (→), Leydig cell (circle), capsule (C), interstitial tissue (It), blood vessel (V). Staining: H-E

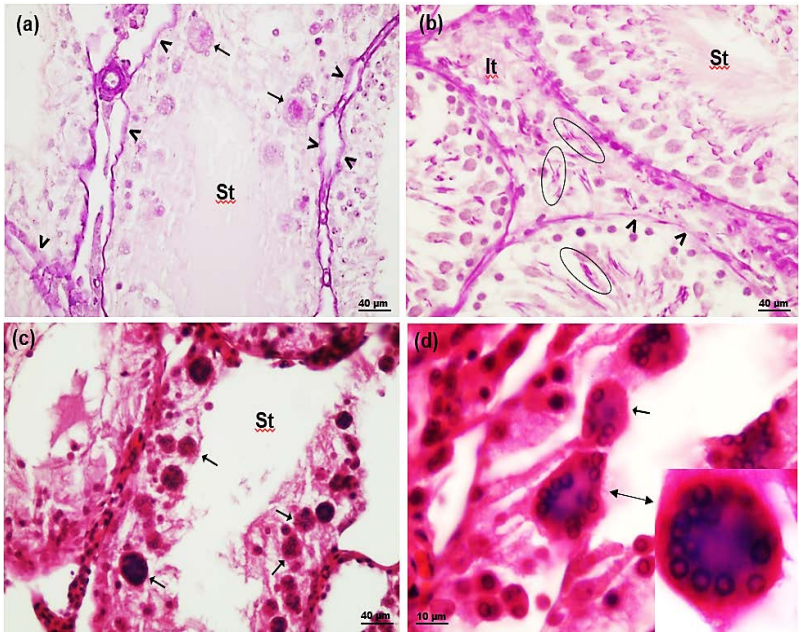

Figure 6. Photomicrographs of the testis of experimental group-2 (G2-0.5 mg/kg/day). a and b; dilatation and decreased PAS-positive staining in the basal lamina of the seminiferous tubules (>), b; sperm-like structures in the interstitial space (oval circle), a, c and d; multinucleated giant cells (→). Seminiferous tubule (St). Staining: a,b: PAS-H, c,d: H-E

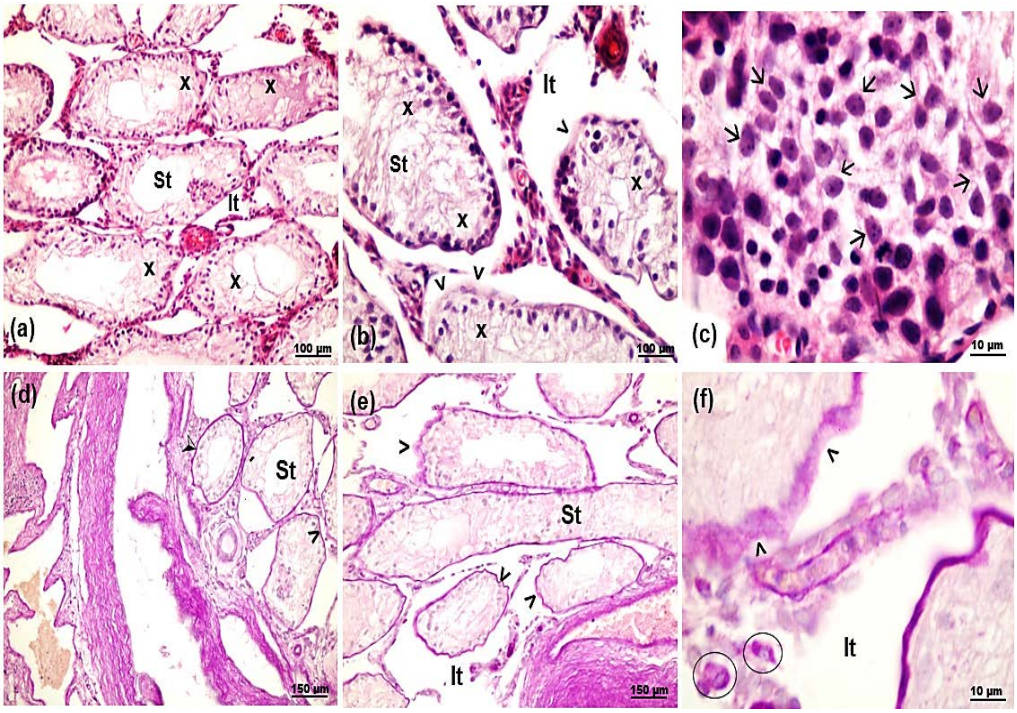

Figure 7. Photomicrographs of the testes of experimental group-3 (G3-1 mg/kg/day). a and b; decrease in spermatogenic cells and disruption of spermatogenic cell arrangement (x), b; loss of seminiferous tubule basal lamina (>), c; increase in Sertoli cells (➔). d; relatively normal in structure and PAS-positive staining basal lamina of the seminiferous tubule (➢), e and f; dilatation and decreased PAS-positive staining in the basal lamina of the seminiferous tubules (>). Seminiferous tubule (St), Leydig cells (circle). Staining: a, b, c: H-E; d, e, f: PAS-H

Morphometric results

In the morphometric analysis in the DiBP-treated groups, a significant decrease ($p < 0.0001$) in seminiferous tubule diameter (STD) and spermatogenic cell line height (SCLH) ($p < 0.0001$) was noted compared to the control group (Table 1). Additionally, there was a dose-dependent significant increase in tubular lumen diameter (TLD) ($p < 0.0001$) and the basal lamina of the seminiferous tubules (BLT) ($p < 0.0001$) after DiBP administration (Table 1). Compared to the control, sham, G1, the increase in basal lamina thickness was quite evident, especially in G3. While multinucleated giant cells (MGC) were not found in control, sham and experimental group-1 (G1), these cells increased significantly in number in G2 and G3. The increase in G2 was particularly striking, but all results for MGC were statistically insignificant ($p > 0.9999$) (Fig. 8, Table 1).

Table 1. Changes in testicular structures after application

	Control	Sham	G1	G2	G3
STD	1058	962.4 ± 95.14	926.1^{a} ± 131.4	544.5^{b} ± 513.0	395.2^{b} ± 662.3
TLD	132.7	140.3 ± 7.594	161.5^{c} ± -28.76	461.6^{b} ± -328.9	724.0^{b} ± -591.3
BLT	20.58	21.86 ± -1.279	24.19 ± -3.603	51.83^{b} ± -31.24	93.89^{b} ± -73.30
SCLH	417.0	401.4 ± 15.68	291.8^{b} ± 125.2	146.1^{b} ± 271.0	163.7^{b} ± 253.3
MGC	0.000	0.000 ± 0.000	0.000 ± 0.000	59.20 ± -59.20	21.40 ± -21.40

[a]: $p = 0.0301$; [b]: $p < 0.0001$; [c]: $p = 0.0058$ (G1: group 1; G2: group 2; G3: group 3).

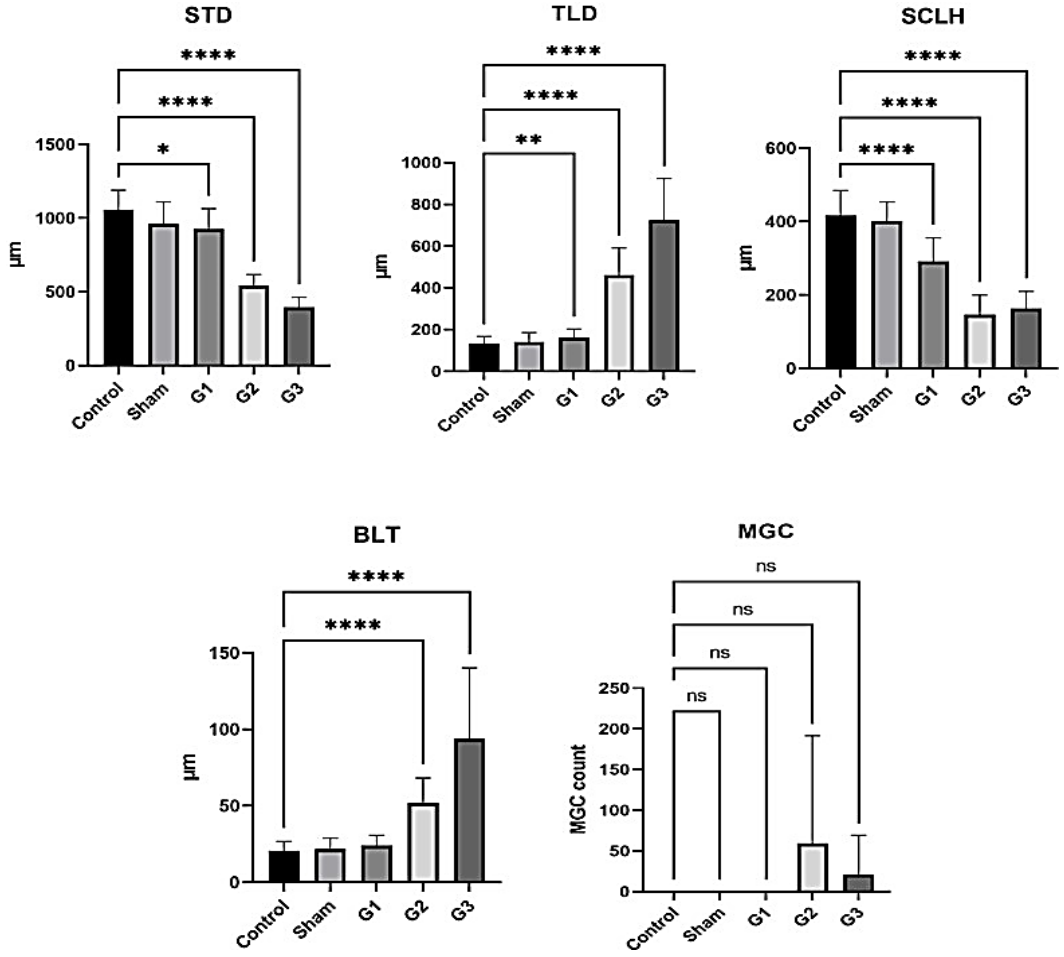

Figure 8. Scoring of three different dose applications of DiBP with pathological parameters. There were significant ($p < 0.0001$) including seminiferous tubule diameter (STD), tubular lumen diameter (TLD), spermatogenic cell line height (SCLH) and basal lamina thickness (BLT) alterations due to dosage, between control and application groups. Compared to control, sham and G1, a statistically non-significant increase in the number of multinucleated cells (MGC) was seen in G2 and G3 ($p > 0.9999$). G1: group 1; G2: group 2; G3: group 3

DISCUSSION

Phthalates are chemical compounds that are widely found in various products and can disrupt the development of male gonads, leading to a condition known as testicular dysgenesis in rats (Mitchell et al., 2012). High exposures to phthalates can occur through medical treatments, such as the use of medical devices containing diethylhexyl phthalate (DEHP) or pharmaceutical drugs with di-n-butyl phthalate (DBP) in their coating (Heudorf et al., 2007). It is important to note that phthalates do not tend to bioaccumulate in the body due to their chemical properties. However, concerns about the developmental and reproductive toxicity of phthalates have been raised based on both animal experiments and human studies, even at ambient concentrations. Published human studies have provided evidence linking phthalate exposure to adverse health effects, including effects on development and reproduction (Duty et al., 2005; Marsee et al., 2006). These studies highlight the potential risks associated with exposure to phthalates and underscore the

importance of minimum risk and harm if humans are exposed to these chemicals.

Spermatogenesis is a specific process of proliferation and differentiation of germ cells that occurs in the seminiferous tubules of the testicles. Seminiferous tubules contain Sertoli cells, which play an important role in supporting and regulating spermatogenesis. Sertoli cells structurally support the germ cells embedded in their cytoplasmic processes and enable their movement from the base to the lumen of the tubule. The Sertoli cells also transfer essential molecules to the substrates for germ cell metabolism and generally regulate the progress of spermatogenesis through to completion. Leydig cells are located in interstitial connective tissue and secrete testosterone hormone (Cheng & Mruk, 2002; Creasy, 2001).

Toxicants acting through a specific cell site generally result in a similar pattern of morphological and physiological changes. Death of specific germ cell populations may be seen in the presence of cytotoxic agents that affect only the mitotic divisions of spermatogonia (Meistrich, 1984; Meistrich, 1986). Li et al. (2022) stated that phthalate (DEHP) exposure in utero can induce testicular dysgenesis syndrome by altering the oxidative balance and SIRT1/PGC1α levels, and taxifolin is an ideal phytomedicine to prevent phthalate-induced testicular dysgenesis syndrome. Similar morphological results were observed in rat testis exposed to DiBP in this study. DiBP exposure disrupted the spermatogenic cell population and caused the complete spermatogenic arrest.

An increase in testicular germ cell death is a commonly reported event after toxic substance exposure. Different germ cell populations (spermatogonia, spermatocytes, spermatids) each display their sensitivity to toxicants. It is rather difficult to say whether germ cell death is due to injury to the Sertoli cell or to direct injury to the germ cell. Detailed and different studies are needed for this. The overriding characteristic of germ cell-specific toxicity is rapid apoptosis and phagocytosis of the affected cells by the Sertoli cell, leaving a tubule depleted of a single generation of germ cells. This early event is then followed by progressive maturation depletion of the descendent cell generations through the rest of spermatogenesis (Creasy, 2001). Sertoli cells, which act as phagocytes when necessary and form the blood testis barrier that prevents the passage of unwanted chemicals, will perform this task to protect the tissue from DiBP. This is a defence mechanism of the tissue and is the cause of the Sertoli cell's survival. However, toxicant-injured Sertoli cells will reduce supportive capacity. Therefore, we can say that the fate of germ cells after Sertoli cell damage caused by the toxic substance will depend entirely on their response to the altered seminiferous tubule environment. Various changes, including decreased secretion of survival factors, increased apoptotic proteins, or a combination of these will result in germ cell apoptosis.

A feature of spermatogenesis is that the dividing spermatogonia and spermatocytes maintain stable cytoplasmic bridges between their progeny, resulting in the interconnection of groups of cells. To some extent, multinucleated giant cell formation may accompany germinal cell degeneration/atrophy. Multinucleated giant cell formation may accompany germinal cell degeneration/atrophy. Normal germ cell division is characterized by incomplete cytokinesis, the new cells formed with each cell division are connected by cytoplasmic bridges. During some forms of degeneration, these cytoplasmic bridges can open and allow the fusion of the cellular contents of the conjoined cells (Creasy & Maronpot, 2020; Creasy et al., 2012). Hild et al. (2007) reported that these were associated with age-related focal testicular atrophy after efferent duct ligation, exposure to gamma radiation or administration of many xenobiotics. These finally die and are probably phagocytized by Sertoli cells or sloughed into the tubular lumen.

Despite all this, Sertoli cells may also be sensitive to chemicals such as phthalates. A recent study showed that (mono-(2-ethylhexyl) phthalate (MEHP) alters Sertoli cell

development during fetal mouse testis cord morphogenesis. Researchers have reported a role for disruption of Retinoic acid (RA) signalling in the mechanisms of MEHP-induced fetal testis toxicity (Alhasnani et al., 2022). One of the most common morphological responses of the Sertoli cell to injury is vacuolization. The phthalate esters, the Sertoli cell cytoplasm show swelling and rarefaction of basal cytoplasm before vacuolation. When examined ultrastructurally, the vacuoles most often appear to represent dilated cisternae of the smooth endoplasmic reticulum (Creasy, 2001). In this study, vacuolization in the cytoplasm of Sertoli cells and decreased PAS-positive reaction in the basal lamina of the tubule were also observed after exposure to DiBP. This situation demonstrates that DiBP damages the organelle structure of Sertoli cells and as a result, they will not be able to perform their duties.

Multinucleated germ cells in seminiferous tubules are a specific form of degenerating germ cell, in contrast to nonspecific germ cell degeneration (Creasy and Maronpot, 2020). It is reported that in-utero exposure to some phthalate esters adversely affects the development of the rat seminiferous cord, causing germ cell loss and increasing the number of multinucleated germ/giant cells (MNGCs) (Spade et al., 2015). Some degree of multinucleated giant cell formation may accompany germinal cell degeneration/atrophy. Normal germ cell division is characterised by incomplete cytokinesis, so the progeny of each cell division are joined to each other by cytoplasmic bridges. During some forms of degeneration, these cytoplasmic bridges can open and allow the fusion of the cellular contents of the conjoined cells (Creasy & Maronpot, 2020).

In this study, multinucleated giant cells of germ cell origin, germinal cell degeneration/atrophy and germ cell loss were also observed in the seminiferous tubules in the high-dose groups despite short-term exposure (28 days). As it is known, substances that pass into the circulation after exposure show their effects by binding to their receptors on the cell membrane or inside the cell in their target cells. The magnitude of this effect depends on the dose of the substance and the amount present in that tissue. Therefore, the greater the dose or concentration of the substance, the greater the effect it creates It was determined that MGCs increased in number in the medium dose group (G2) and were less in the highest dose group (G3) compared to G2 in this study. Although the concentration of the substance increased, its effect did not increase after a certain level. We can explain this situation as follows; there is no empty receptor left in the cell where DBP can exert its effect. In short, most receptors have been affected by DIBP molecules. Despite increasing the dose of the substance, no further biological response has occurred.

The endothelium of rodent testicular capillaries may be sensitive to certain substances/chemicals, resulting in massive interstitial oedema and cessation of blood flow. Interstitial fluid, produced as a transudate from the capillaries, and seminiferous tubule fluid, secreted by the testis, can be readily disturbed, resulting in various effects, including tubular lumenal dilatation, tubular lumenal contraction, and interstitial oedema (Setchell, 1990). In the experimental groups of this study, thickening and enlargement of the basal lamina of the seminiferous tubules, oedema and shrinkage of the interstitial connective tissue were determined. DIBP, like some other chemicals, may have disrupted blood flow and fluid balance by causing changes in testicular structure. The basal and underlying collagen network may even facilitate cross-talk between the seminiferous epithelium, the myoid cells and cells in the interstitium. Studies have illustrated the crucial role of the extracellular matrix in Sertoli and germ cell function in the seminiferous epithelium, including the blood-testis barrier dynamics (Cheng & Siu, 2008). In our previous study, a decrease in the localisation of the immunoreactivity of fibronectin and laminin, which are ECM components that play an important role in spermatogenesis, was observed after DIBP application, suggesting

that this may be the cause of infertility (Başımoğlu Koca, 2019).

In this study, the expansion and decreased PAS-positive reaction in the basal lamina of the seminiferous tubule after DiBP application showed that the blood-testis barrier was disrupted and DIBP passage was facilitated. As a result, the spermatogenic cell line was negatively affected and even reduced naturally. In summary, DiBP exposure caused germ cell death, decreased seminiferous tubule diameter, increased tubular lumen diameter, basal lamina thickness and the induction of MGCs. This was also supported morphometrically in this study.

CONCLUSION

This study showed that DiBP caused irreversible damage to the testicular tissue of experimental animals. Although the evaluation of this issue needs to be done through different studies, it is possible to say that the substance in question may cause infertility in men, according to the results of this research. Therefore, we recommend using an alternative plasticiser with proven safety instead of DiBP. Although the negative effects of phthalate exposure have been more or less understood in recent years, such substances still maintain their importance and seriousness in terms of human health and the environment. Therefore, together with previous studies, future studies will contribute to elucidating the details of phthalates and better understanding their effects.

Acknowledgements: This study was funded by Adnan Menderes University Scientific Researcher Project Unit [Number: FEF-14018]. The author thanks Merve Akyıldız for her help in caring for the animals and processing the tissues and is extremely grateful to Dr. Fatih Çöllü for his contributions to the statistical analysis.

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