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Male rat exposure to low dose of di(2-ethylhexyl) phthalate during pre-pubertal, pubertal and post-pubertal periods: Impact on sperm count, gonad histology and testosterone secretion

Malha Oudir[a,*], Henni Chader[b], Bachir Bouzid[a], Kheira Bendisari[c], Batoul Latreche[c], Sofiane Boudalia[d], Mokrane Iguer-ouada[e]

[a] Laboratoire de Génie Chimique, Département de Génie des Procédés, Faculté de Technologie, Université Saâd Dahlab, USDB. BP 270, Route de Soumâa, 09000 Blida, Algeria
[b] Département de Pharmacie, Faculté de Médecine, Université d'Alger 1, Algeria
[c] Département d'Anatomie et Cytologie Pathologique, Hôpital Hospitalo-Universitaire Beni Messous, Algeria
[d] Laboratoire de Biologie et/ou Environnement Département d'Ecologie et du Génie de l'Environnement, Université 8 Mai 1945, Guelma, Algeria
[e] Département de l'Environnement et Sciences Biologiques, Faculté des Sciences de la Nature et de la Vie, Université A. Mira, 06000, Bejaia, Algeria

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ABSTRACT

Di (2-ethylhexyl) phthalate (DEHP) is the most ubiquitous endocrine disruptor in the environment. The present study aimed to investigate the low dose effects of DEHP on the male reproductive system of rats exposed during the pre-pubertal, pubertal and post-pubertal periods. Male Wistar rats were daily gavaged by DEHP from postnatal day (PND) 21 to PND 120 with 0.5, 50 and 5000 μg/kg BW/d. A decrease in sperm count of 41%, 24% and 46% was observed at 0.5, 50 and 5000 μg/kg BW/d respectively. A decrease of Sertoli cells number was observed at 50 and 5000 μg/kg BW/d (22% and 42%, respectively). Non-monotonic dose–response was observed for testosterone levels with a significant increase at 50 μg/kg BW/d associated to a notable enhancement of Leydig cells number (35%). In conclusion, our results showed that postnatal exposure to low doses of DEHP affects sperm count, Sertoli and Leydig cells number and testosterone level.

1. Introduction

Di-(2-ethylhexyl) phthalate (DEHP) is a plasticizer produced by phthalic acid esterification reaction with 2-ethylhexanol in presence of an acid catalyst [1,2]. DEHP is mainly added to polyvinyl chloride to make it smooth, flexible and durable, so it is found in most everyday consumer products such as food packaging, toys, medical devices, construction products and clothing; plastics can contain up to 40% DEHP [3–5].

Due to the absence of covalent chemical bonding to the matrix polymer, DEHP is constantly released into the environment by migration and evaporation, resulting in its becoming an ubiquitous environment contaminant [6,7]. Total emission into the environment from EU countries is estimated at 28 653 t [8].

Consequently, the general population is constantly exposed to DEHP through multiple routes and sources of exposure: orally *via* food and water, respiratory *via* air, dermally *via* cosmetics, parenterally and intravenously *via* medical devices and placenta during pregnancy [7,9]. Vandenberg et al. [23] estimated that human exposure to DEHP to be 0.5–25 μg/kg BW/d.

DEHP has been recognized as an endocrine disruptor and associated with reproductive toxic effects [3–5]. However, a tolerable daily intake (TDI) of 50 μg/kg BW/d was established by the European Food Safety Authority (EFSA) in 2005 based on the "no adverse effect level" (NOAEL) of 5 mg/kg BW/d evaluated by Wolfe and Layton [10].

The majority of studies concerning DEHP were carried out with high doses during short exposure periods, and the reproductive adverse effects following postnatal exposure of male animals has been well-documented. Severe disorders were reported including decrease in sperm count and motility, cessation of spermatogenesis, decrease in fertility index, diminished testosterone, decreased testis and epididymal weights with severe lesions in the testes

* Corresponding author.
E-mail address: oudirmalha@univ-blida.dz (M. Oudir).

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including: Leydig cell hyperplasia, atrophy of seminiferous tubules with a progressive degeneration of germ cells, mostly spermatocytes and spermatids, which ultimately slough off into the tubular lumen [2,6,11–20].

In the recent years, it was demonstrated that certain EDCs had produced toxic effects at even very low dose exposure during "critical windows" [21–25]. By definition, "low doses" are doses below the NOAEL set in traditional toxicology studies, or doses in the range of typical human exposure in the environment [26]. Thus, Low-dose effects are effects observed at doses below the NOAEL or effects that occur in the typical human exposure range according to the National Toxicology Program [27].

Several low-doses studies dedicated to EDCs demonstrated that the period of exposure is determinant, thus early periods of development (*in utero*, pre-pubertal and pubertal) are more sensitive to the effects of EDCs exposure [28]. Consequently, the traditional concept in toxicology changed from "dose makes the poison" to "timing makes the poison" [28,29].

In this respect, the period of male reproductive tract development was demonstrated to be the most sensitive to testicular toxicity by DEHP [30,31]. To the best of our knowledge, the only study dedicated to investigate the low-dose effects of postnatal exposure of DEHP in male rats, was conducted on pubertal to post-pubertal periods. Males were daily gavaged with 30, 100, 300, and 1000 μg/ kg BW/d of DEHP from postnatal day (PND) 42 to PND 105; no significant differences were observed in sperm counts, motility and relative testes and epididymis weights [32]. Other studies using intermediates and high DEHP doses covered longer periods starting particularly at weaning to adulthood (from PND 21 to PND 120). The results are controversial concerning testosterone and a decrease in epididymal sperm counts is observed only at 900 mg/kg BW/d [12,33]. To the best of our knowledge, the low doseeffects ofDEHPduring this period of PND 21 to PND 120 have never been investigated.

Based on the presented background, the aim of the current study is to evaluate low dose effects exposure to DEHP from weaning to adulthood, (PND 21 to PND 120) in male rat. This period of treatment was chosen as in the environment the exposure to DEHP is constant during the different periods of life being, so the rationale behind this duration is to mimic the continuous ambient low-level exposures to DEHP. Different parameters were investigated, including: sperm count and motility, testosterone levels and histopathological examination of testis and epididymis. Three DEHP doses were tested: the lowest dose of human exposure (0.5 μg/kg BW/d), the regular doses considered to be a 'safe dose' for humans (50 μg/kg BW/d) and a dose considered to be NOAEL for reproductive development in the rat (5000 μg/kg BW/d).

2. Material and methods

2.1. Chemicals

DEHP, CAS N° 117-81-7, purity 99.8% was obtained from LG CHEMICAL, its identity confirmed with analytical techniques. DEHP was dissolved in olive oil which was used as the vehicle; the dosing solutions were kept in glass bottles in the dark, at room temperature, and continuously stirred during the dosing period. All of the products complied with the quality standards of the European Pharmacopoeia (http://www.edqm.eu/en/Homepage-628.html).

2.2. *Animal treatment and housing conditions*

All procedures involving rats were approved by the local authorities, and adhered to the ethical guidelines for the care and use of laboratory animals.

Forty SPF (specific pathogen-free) male Wistar rats (obtained from Charles River Laboratory, France) were randomly divided into four 4 groups of 10 rats each, receiving DEHP dosages of 0; 0.5 μg/kg BW/d; 50 μg/kg BW/d and 5000 μg/kg BW/d respectively, *via* daily gavage with a stainless steel probe from PND 21 to PND 120. The control group received the vehicle alone, dosing volume was 10 ml/kg, adjusted on a daily basis according to body weight. Rats were housed individually in transparent polycarbonate cage type E (Charles River) in experimental animal room maintained at 21 ± 5 °C, 53 ± 7% humidity and 12 h light dark photocycle. Food pellets and water filtered by activated carbon in glass bottles were available *ad libitum*. Rats were daily inspected for general toxicity and killed by decapitation at PND 120. In order to avoid eventual effects due to stress, the same experimental conditions were kept during the entire treatment period, and all plastic material was removed.

2.3. Body weight, euthanasia and reproductive organ weights

At PND 120 all rats were weighed and killed by decapitation, blood was collected in glass tubes and immediately placed on ice. Soon after euthanasia and blood collection, testes and epididymides were removed and weighed. The right cauda epididymides was weighed and placed in the pre-labeled glass Petri dish containing 10 ml of pre-warmed bovine serum albumin/phosphate buffered saline (BSA/PBS) (1% BSA) and incubated at approximately 34 °C and then used for sperm analysis. The contralateral testis and epididymis were fixed in 10% formalin and used for histopathological examination.

2.4. Epididymal sperm count and motility analysis

The procedure used for epididymal sperm count and motility analysis was based on Parker protocol [34]. Immediately after euthanasia, the right cauda epididymis was weighed and placed in a pre-labeled glass Petri dish containing 10 ml of prewarmed BSA/PBS diluent (1% BSA). The cauda epididymis was then pierced four to six times using a scalpel blade. The glass Petri dishes were covered and incubated at 34 °C for 8 min to allow for sperm swimout. In order to estimate the percentage of motile sperm, 10 μl aliquot was placed in a Malassez chamber and analyzed under a phase-contrast microscope (HundWetzlar ×40 objective). At least 200 sperm were evaluated per animal and classified as mobile or immobile. Motility was expressed as a ratio of number of motile sperm to the total number of sperm. To assess the concentration of spermatozoa, sperm aliquot was diluted 1:10 with fixative (1% formalin in PBS) and counted using a Malassez chamber. Sperm count is reported as the sperm number per gram of relative weight of cauda epididymis.

2.5. Histopathologicalexamination

After fixation in 10% formalin solution, testes and epididymis were transferred to different increasing concentration of alcohol baths (70%, 90%, 100% ethanol), embedded in paraffin and cut at 3 μm. Sections were stained with hematoxilin and eosin and evaluated by light microscopy (LEICA DM LS2 Microsystems). Histopathological evaluation was performed by a specialist in histopathology in a blind-manner. The analysis has mainly focused on the qualitative study of spermatozoa in the epididymal duct from the proximal caudaepididymis andin lumen of the seminiferous tubules. On the basis of three (03) different cross-sections per animal, a total of one hundred eighty (180) random tubules were analyzed per group (n = 10/group). The seminiferous epithelium cycle was globally evaluated by the presence of spermatozoa and the various germ cells: spermatogonia, spermatocytes and sper-

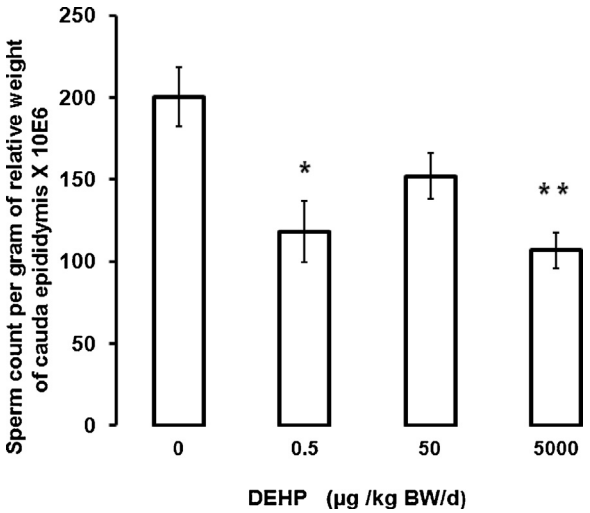

Fig. 1. Low doses effects of DEHP exposure from PND 21 to PND 120 on rats sperm count. Each bar represents the mean ± SE of sperm count per gram of relative weight of cauda epididymis. Means which are denoted by *, ** differ from the control group (ANOVA, $p < 0.05$, $p < 0.01$) (n = 10/group).

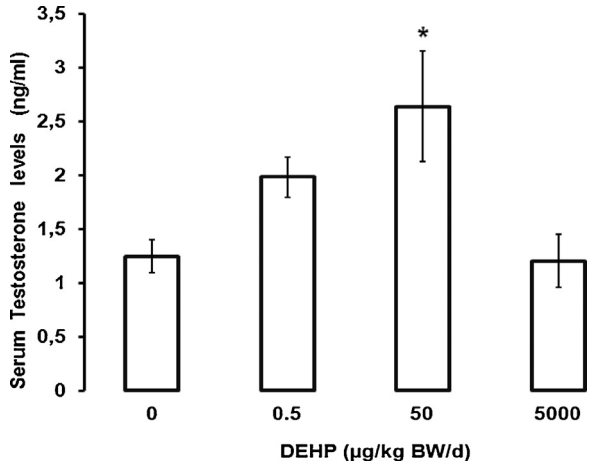

Fig. 2. Effect of different low doses of DEHP exposure from PND 21 to PND 120 on rats Testosterone levels. Each bar represents the mean ± SE of serum testosterone levels (ng/ml). Means which are denoted by * differ from the control group (ANOVA, $p < 0.05$) (n = 10/group).12) at 5000 µg/kg BW/d. No effect was observed at 0.5 µg/kg BW/d.

matids in examining seminiferous tubules. The oligospermia was estimated based on the quantity of spermatozoa in the lumen of seminiferous tubules and in the proximal cauda epididymis. Severe oligospermia (+), moderate oligospermia (++) and normal spermatozoa quantity (+++) corresponded to the presence ofspermatozoa in 25%, 50% and >80% of the analyzed tubules. Leydig cells were counted in 10 different fields per animals and Sertoli cells were counted in 5 random seminiferous tubules per animals (n = 10) under a light microscope (DM750 LEICA microsystem) at ×400 magnification.

2.6. Serum testosterone concentration

The collected blood was centrifuged at 4000 rpm for 5 min at 4 °C, the resulting serum was then transferred to polypropylene tubes and stored at −20 °C for further analysis. Serum testosterone concentration was measured by Electrochemiluminescence immunoassay (ECLIA) (**IMMUNO ANALYSES COBAS ROCHE).** The limit of detection was 0.025 ng/ml.

2.7. Statistical analysis

Statistical analysis was realized using SPSS software (version 2.1), results were expressed as mean ± SE. Means were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test to determine significant differences among the groups. Two linear regression models were used to test the non-monotonicity: a simple linear regression model and a regression model that included a quadratic term. Differences were considered to be statistically significant at a probability level of 5% ($p < 0.05$). Correlation test was performed to evaluate the relationships between the different measured parameters including sperm count, testis and epididymis weights and histopathological parameters.

3. Results

3.1. Sperm count

The current results showed that Wistar male rats exposure to low doses of DEHP from pre-pubertal to post-pubertal (PND 21 to PND 120) induced a significant decrease in sperm count compared to the control group (ANOVA p = 0.01) (Fig. 1). Indeed, a 46%, 41% and 24% decrease at 5000 µg/kg BW/d, 0.5 µg/kg BW/d and 50 µg/kg BW/d was observed, respectively. Surprisingly, a non-linear relationship was observed between the used doses and sperm counts. In fact, the intermediate dose (50 µg/kg BW/d) did not decrease sperm count to the same extent as the lowest dose (0.5 µg/kg BW/d).

3.2. Sperm motility

Compared to sperm count, sperm motility does not appear to be significantly affected by prepubertal, pubertal and pospubertal exposure to low doses of DEHP (ANOVA p = 0.3). When compared to the control group, a decrease of 29%, 18.63% and 9% was observed at 5000 µk/kg BW/d, 50 µg/kg BW/d and 0.5 µg/kg BW/d, respectively (Table 1).

3.3. Testosterone levels

The level of testosterone measured at PND 120 (Fig. 2), was significantly affected by exposure to low doses of DEHP (ANOVA p = 0.006). Indeed, a significant increase in testosterone level (2.64 ± 0.5 ng/ml) was noticed at the intermediate tested dose (50 µg/kg BW/d), (p = 0.01) compared to the control (1.24 ± 0.15 ng/ml). The highest testeddose (5000 µg/kg BW/d) did not alter the values of testosterone levels – because the value was similar to control, suggesting no alteration in this parameter. On the other hand, at the lowest tested dose (0.5 µg/kg BW/d), a slight increase was observed (1.98 ± 0.18 ng/ml), although not statistically significant (p = 0.34) compared to the control. Thus, when analyzing the effect on testosterone of all tested doses, an inverted-U dose–response relationship was observed. In order to verify this model, the results were analyzed using two regression models identified in the methods section. The simple linear regression model gave a p = 0.9 and a low coefficient of determination $R^2 = 0.01$, meaning that this linear model is invalid. However, when the quadratic term was added to the linear model, a p value = 0.3 and $R^2 = 0.85$ were achieved, expressing that this quadratic model fits better with the results.

3.4. Body and relative organ weights

Exposure to low doses of DEHP from PND 21 to PND 120 did not affect body weight in all treated groups(ANOVA p = 0.14) (Table 1).

Table 1
Body weight, absolute and relative organ weights (testes, epididymis) and sperm motility percentageat PND 120 in male rats following prepuberty, pubertal and postpubertal exposure to low doses of DEHP.

Parameter	DEHP doses (μg/kg BW/d)			
	0 (*n* = 10)	0.5 (*n* = 10)	50 (*n* = 10)	5000 (*n* = 10)
Body weight (g)	343.7 ± 6.40	314.4 ± 9.04	332.9 ± 9.36	323.9 ± 10.91
Absolute Testis weight	1.64 ± 0.047	1.54 ± 0.063	1.39 ± 0.055*	1.11 ± 0.079**
Absolute Epididymis weight	0.27 ± 0.006	0.23 ± 0.009*	0.20 ± 0.005**	0.18 ± 0.007**
Relative Testis weight (g)	0.41 ± 0.014	0.49 ± 0.016*	0.42 ± 0.018	0.34 ± 0.019*
Relative Epididymis weight (g)	0.068 ± 0.001	0.074 ± 0.002	0.062 ± 0.002	0.057 ± 0.001**
Sperm motility (%)	44.55 ± 3.17	40.37 ± 3.39	36.25 ± 3.51	33.59 ± 6.27

Results are expressed as mean ± SE. (ANOVA $p < 0.05$).
* Significantly different from control group, $p < 0.05$ (Tukey test).
** Significantly different from control group, $p < 0.01$ (Tukey test).

A significant dose-related decrease in absolute testis and epididymis weights were observed in all treated groups compared to control. Absolute testis weight decreased by 6% ($p = 0.68$), 15% ($p = 0.04$) and 32% ($p = 0.000$) at 0.5, 50 and 5000 μg/kg BW/d, respectively. Similarly, absolute epididymis weight decreased by 14% ($p = 0.005$), 25% ($p = 0.000$) and 33% ($p = 0.000$) at 0.5, 50 and 5000 μg/kg BW/d, respectively. The effect on the relative epididymis weight was significant only at 5000 μg/kg BW/d with a decrease of 16% ($p = 0.001$). Concerning relative testis weight, we observed a significant increase (19%) at 0.5 μg/kg BW/d and inversely a significant decrease (17%) at 5000 μg/kg BW/d. The intermediate dose (50 μg/kg BW/d) appeared to have no effect on relative testis and epididymis weights (Table 1). These results also indicated a non-linear response between doses and relative organ weights. In the case of relative testis weights, the simple linear model gave $p = 0.41$ (not statistically significant) and a low $R^2 = 0.34$, and the quadratic model gave $p = 0.29$ and $R^2 = 0.91$ indicating that the non-linear model fitsbetter the data than the simple linear model.

3.5. Histopathologic examination

Histopathological examination of the epididymis revealed results that are consistent with those observed in the spermogram and caudaepididymal sperm counts (Table 2, Fig. 3).

In fact spermatozoa were partially or totally absent depending on the tested dose; 8 out of 10 cases of oligospermia, including2 severe cases, were observed at the highest dose (5000 μg/kg BW/d, Fig. 3D), 6/10 cases of moderate oligospermia, at the intermediate dose (50 μg/kg BW/d, Fig. 3C) and 6/10 cases of oligospermia, with 2 severe cases, at the lowest tested dose (0.5 μg/kg BW/d, Fig. 3B). No cases of azoospermia were observed in all treated groups (Table 2, Fig. 3).

In testes,at 5000 μg/kg BW/d as well as at the lowest tested dose (0.5 μg/kg BW/d), mature spermatozoa were present in small amounts or totally absent depending on the analyzed seminiferous tubules (Fig. 3H and F, respectively) with one case of vacuolar degeneration at each of these doses. While, the intermediate dose (50 μg/kg BW/d) tubules lumen were full of mature spermatozoa or totally absent depending on the analyzed seminiferous tubules (Fig. 3G). Spermatid cells were present in nearly 80% all analyzed groups (Table 2). Therefore, the different stages of spermatogonia and spermatocytes were observed in all examined seminiferous tubules.

Compared to the control, Sertoli cells number showed a significant dose-related decrease in the treated groups with 22% ($p = 0.03$) and 42% ($p = 0.000$) at 50 and 5000 μg/kg BW/d respectively. Leydig cells number was also affected by treatments with a significant increase (35% ($p = 0.03$)) at 50 μg/kg BW/d and a decrease (21%) (not statistically significant, $p = 0$

4. Discussion

The concept of low-dose effects of EDCs is currently at the center of scientific debates resulting in a great controversy regarding the chemical risk assessment. In fact, in recent years several studies of EDCs have challenged the traditional concept in toxicology which stipulates “the dose makes the poison”, as EDCs can express adverse effects at very low doses but not necessarily at higher doses [23], particularly during “critical windows” [21–24].

This study aimed to evaluate the low dose effects exposure to DEHP, anubiquitous endocrine disruptor in the environment, during the most sensitive periods of the development of the reproductive system corresponding to pre-pubertal, pubertal and post-pubertal periods. Different reproductive parameters were investigated including sperm count, testosterone levels and histopathological evaluation of testes and epididymis.

The results of the current study showed that pre-pubertal, pubertal and post-pubertal exposure to low doses of DEHP (5000, 50 and 0.5 μg/kg BW/d) decreased significantly the sperm counts, by 46, 24 and 41% respectively. This decrease in sperm count is tightly related to the oligospermia findings in the histopathological examination and correlated positively with absolute testis and epididymis weights ($r = 0.53$ and $r = 0.60$, respectively). Similar results were reported at higher doses of DEHP (from 250 to 2000 mg/kg BW/d), during a short exposure period of 10–15 days only, in adult SD rats [19,20,35]. The current results are also consistent with those observed by Zhang et al. [36] at low doses in mice, reporting a decrease in sperm count after exposure to 5, 20 and 40 μg/kg BW/d of DEHP during neonatal period (PND 7 to PND 49). On the other hand, in rats, Hsu et al. [32] observed no significant effects on sperm counts following pubertal and post-pubertal (PND 42 from PND 105) exposure to 30, 100, 300, and 1000 μg/ kg BW/d of DEHP considered as low doses. This is probably related to the fact that rats are not exposed during the pre-pubertal period which characterized by an active proliferation of Sertoli cells [38,41]. In the current results, Sertoli cells counting revealed a significant decrease (22% and 42% at 50 and 5000 μg/kg BW/d, respectively) impacting consequently and negatively sperm count ($r = 0.18$). This is previously reported in several studies investigating pre-pubertal exposure to high doses of DEHP [42,30,41]. On the other hand, at the lowest dose (0.5 μg/kg BW/d, which is a very low dose)despite the decrease in sperm count, Sertoli cells number was not affected, a similar result was reported by Andrade et al. [43] after in utero exposure. This is probably related to the fact that DEHP interferes with different and multiple mechanisms of action depending on the period and the dose level, as indicated by Schug et al. and Lyche et al., [22,44]. Zhang et al. [36], have suggested that at low dose, the decrease in sperm quality, including sperm count, is due to reduced expression of genes: DDx3Y, Usp9Y, RBM, E1F1AY, EGF, FSHR and EGFR. Parmar et al. [19] suggested also that DEHP affect sperm count by altering the activities of the enzymes

Table 2
Histopathologic lesions in the epididymis and testis at PND 120 in male rats following pre-puberty, pubertal and post-pubertal exposure to low doses of DEHP.

Parameter		DEHP doses μg/kg BW/d			
		0 (*n* = 10)	0.5 (*n* = 10)	50 (*n* = 10)	5000 (*n* = 10)
Epididymis					
Dispersion	Scattered	0	7	7	10
	Not scattered	10	3	3	0
Azoospermia		0	0	0	0
Severe oligospermia (<25%)		0	2	0	2
Moderate Oligospermia (~50%)		1	4	6	6
Dense (>80%)		9	4	4	2
Testes					
Seminiferous tubules					
Vacuolar Degeneration		0%	10%	0%	10%
Sertoli cell number ♀eminiferous tubule		13.6 ± 0.77	12.8 ± 0.68	10.6 ± 0.84*	7.80 ± 0.69**
Spermatids		>80%	>80%	>80%	>80%
Spermatozoa		>80%	<50%	~60%	<25%
Interstitial tissue					
Leydig Cell number/field		12.20 ± 0.61	11.5 ± 0.63	16.5 ± 1.03*	9.6 ± 0.85

Sertoli and Leydig cells number results are expressed as mean ± SE. (ANOVA $p < 0.05$).
* Significantly different from the control group, $p < 0.05$ (Tukey test).
** Significantly different from the control group, $p < 0.01$ (Tukey test).

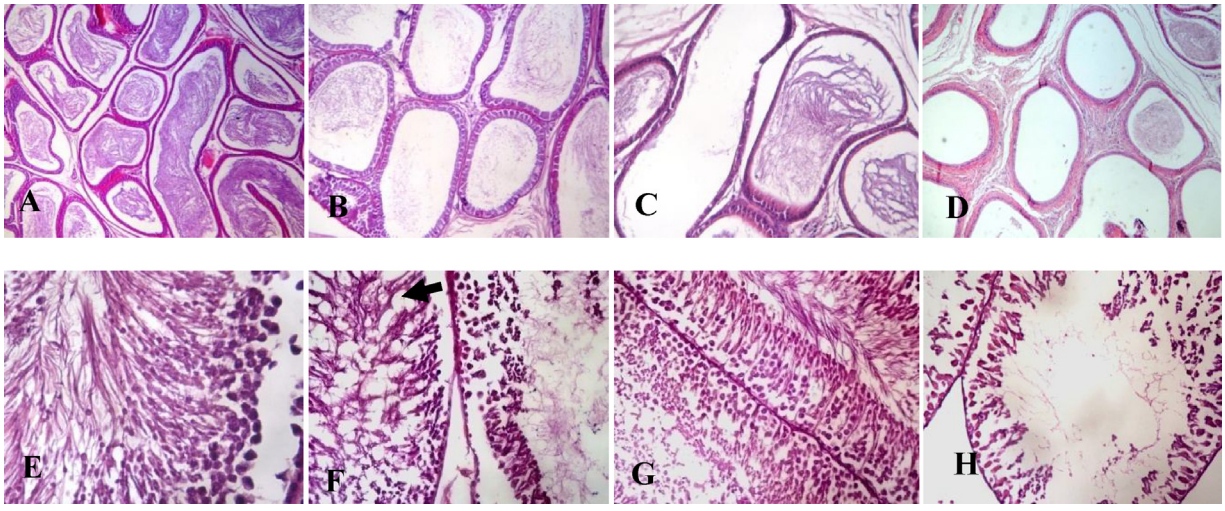

Fig. 3. Histological lesions in the epididymis and testis of rat at PND 120 following pre-puberty, pubertal and post-pubertal exposure to low doses of DEHP. **A**: (4×) Proximal cauda epididymis of control rat, Epididymal duct is full of spermatozoa. **B** and **C**: (10×) Proximal cauda epididymis of rats exposed to 50 and 0.5 μg/kg BW/d respectively, epididymal duct is fully or partially full of spermatozoa. **D**: (10×) Proximal cauda epididymis of rat exposed to 5000 μg/kg BW/d, spermatozoa are partially or totally absent in epididymal duct. **E**: (40×) Testis of control rat. **F:** (20×) testes of rats exposed to 0.5 μg/kg BW/d, mature spermatozoa are present in small amounts with a simultaneous vacuolar degeneration (arrow); G: (20×) Testes of rats exposed to 50 μg/kg BW/d lumen is full of mature spermatozoa or totally absent. **H**: (20×) Testis of rat exposed to 5000 μg/kg BW/d, mature spermatozoa are partially or totally absent.

responsible for the maturation of sperms.This is in agreement with the current results as histopathological examination revealed the presence of spermatids in all examined seminiferous tubules suggesting an impacton spermatozoa maturation. Indeed, according to Holson et al. [40], a normal number of spermatids and a reduction in epididymal sperm count implies that sperm maturation is affected. Similarly, Dostal et al. [30] reported a dose related decrease in maturation of the spermatids after 6 weeks of exposure to 10, 100, 1000 and 2000 mg/kg of DEHP.

The results of testosterone levels revealed non-monotonic dose–response with a significant increase at the intermediate dose 50 μg/kg BW/d. To the best of our knowledge, the current study is the first to be dedicated to investigate the effects at low doses of DEHP on testosterone during postnatal exposure. The few studies carried outpreviously at low doses, have been focused on the *in utero* exposure, and similarly to the current study, they revealed non monotonic dose-responses relationships in rat [43] and mouse [45]. This non-monotonic dose–response relationships,often referred to as biphasic dose-response curves, and low-dose effects of EDCs have been described in the literature in recent years. Vandenberg [46] indicated that non-monotonic dose response curves have been demonstrated for EDCs and the mechanism responsible is related to the interactions between the ligand and hormone receptors. In March 2012, Vandenberg et al. published a review on low-dose effects and non monotonic dose responses of EDCs, they indicated that low doses of EDCs give non-monotonic responses with inverted U sharp and concluded that "when non monotonic dose-response curves occur, the effects of low doses cannot be predicted by the effects observed at high doses". A similar biphasic dose-response model have been also reported by Ge et al. [47] which stipulates that low doses of DEHP increase the testosterone level, whereas high doses reduce testosterone level. In their study, male rats were treated with 10, 500, and 750 mg/kg BW/d DEHP during pre-pubertal period from PND 21 to PND 48. The results showed a significant increase in testosterone levels (3.13 ± 0.37 ng/m) at 10 mg/kg BW/d com-

pared tothe control (1.98 ± 0.20 ng/ml), while the 750 mg/kg BW/d dose decreased testosterone (1.18 ± 0.18 ng/ml). The same authors tested the DEHP metabolite MEHP *in vitro* in Leydig cell cultures, and they showed that 27.8 μg/ml of MEHP increased testosterone production, while 2780 μg/ml was inhibitory. Similarly, Akingbemi et al. [12] reported a significant increase in testosterone levels following pre-pubertal to post-pubertal exposure (from PND 21 to PND 120) at both doses 10 mg/kg BW/d and 100 mg/kg BW/d in rat. In the opposite, at high doses (500, 750, 1000, 1250 mg/kg BW/d)the majority of studies reported an anti-androgenic action of DEHP [11,14,17,35,47–49].

In the current study, the increased testosterone level was associated to a notable enhancement of Leydig cells number. In fact, Leydig cells increased by 35% at 50 μg/kg BW/d suggesting a direct effect on the testosterone level. A similar mechanism of action has been reported by Akingbemi et al. [12] during the same periods of exposure (from PND 21 to PND 120) using 10 and 100 mg/kg BW/dDEHP. At the lowest tested dose (0.5 μg/kg BW/d),the increased testosterone level (not statistically significant) was not associated to increased Leydig cells number. Akingbemi et al. [50] have reported a similar result and according to these authors, this is probably due to a compensatory mechanism. At high doses, the majority of studies linked the systematic testosterone decrease to the degeneration of Leydig cells and to the inhibition of testosterone-biosynthetic enzyme [48–50].

In conclusion, the present study revealed that exposure to low doses DEHP during pre-pubertal, pubertal and post-pubertal periods (from PND 21 to PND 120), affected sperm counts, Sertoli and Leydig cells number and testosterone levels. The dose of 5000 μg/kg BW/d, considered to be NOAEL for reproductive development in rat, decreased sperm count and Sertoli cells number by 46% and 42%, respectively. The lowest tested dose (0.5 μg/kg BW/d); which is a hundred times lower than the TDI, decreased also sperm count and Sertoli cell number by 41% and 6%, respectively. The dose of 50 μg/kg BW/day, considered to be a 'safe dose' for humans, decreased sperm count by 24% and Sertoli cell number by 22%. Concerning testosterone levels, the results showed non-monotonic dose–response with maximum levels obtained at the intermediate tested dose (50 μg/kg BW/d) associated to an increase in Leydig cells number. Thus, our results showed that exposure to low doses of DEHP, far below or at regulatory doses, from pre-pubertal to pos-pubertal periods, disrupt significantly the reproductive system, and indicated that the concept of "safe dose" estimated from the NOAEL is not valid for DEHP.

Competing interest

The authors have declared that no competing interest exists.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.reprotox.2017.11.004.

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