REVIEW

Reproductive toxicity of phthalate esters

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Phthalate esters are ubiquitous environmental contaminants that in general display low-toxicity. Overall, the reproductive effects of these compounds are well characterized in adult's animals, with gonadal injury observed after high dose exposure. However, results of recent transgeneration studies indicate that the reproductive system of developing animals is particularly vulnerable to certain phthalates. The phenotypic alterations observed in male offspring rats exposed during the perinatal period have remarkable similarities with common human reproductive disorders, including cryptorchidism, hypospadias and low-sperm counts. Recent results also indicate that high phthalate doses can adversely affect adult and developing female rats. However, the main question involving phthalates is whether the current level of human exposure is sufficient to adversely affect male and/or female reproductive health. Here, we review the reproductive toxicity data of phthalates in adult and developing animals as well as possible modes of action. In addition, we briefly discuss the relevance of animal studies to humans in light of recent epidemiological data and experimental research with low (human relevant) doses. Finally, we point out some critical issues that should be addressed in order to clarify the implications of phthalates for human reproduction.

Keywords:
Human reproductive health / Low-dose effects / Phthalates / Reproductive and developmental toxicity / Testicular dysgenesis syndrome

1 Introduction

Phthalates, dialkyl- or alkyl/aryl-esters of phthalic acid, are industrial chemicals used primarily as plasticizers to impart flexibility to polyvinylchloride plastics. They are present in a wide variety of products, including building materials, food packaging, clothing, toys and medical devices. Di-(2-ethylhexyl) phthalate (DEHP) is currently the most commonly used phthalate plasticizer for polyvinylchloride worldwide. In addition, some other phthalates are used as additives in cosmetics, pharmaceuticals, lubricant oils and solvents [1, 2].

Phthalates are not covalently bound to the plastic matrix and can easily leach out to contaminate the external environment [3, 4]. It is not surprising that DEHP and other phthalates can be found in several media including food, water, infant formula, house dust and air [4–6]. The major pathway of human exposure is through ingestion of contaminated food and water but other routes including inhalation and dermal contact may also contribute to the measured body burden of phthalates [1]. The widespread exposure of the general human population has been demonstrated in several recent biomonitoring studies in the USA and Europe [7–9].

Although most phthalates show very low-acute toxicity, experimental data indicate that the reproductive system is particularly susceptible to these compounds. Currently, there are specific regulations in Europe, which restrict the use of reproductive toxic phthalates in toys, cosmetics and food contact materials. In 2005, the European Union

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Abbreviations: BBP, butyl benzyl phthalate; **DBP,** dibutyl phthalate; **DEHP,** di-(2-ethylhexyl) phthalate; **DEP,** diethyl phthalate; **Insl 3,** insulin-like factor 3; **MBP,** mono-*n*-butyl phthalate; **MEHP,** mono-(2-ethylhexyl) phthalate; **PPAR,** peroxisome proliferator-activated receptor; **TDS,** testicular dysgenesis syndrome

approved the ban of reproductive toxic phthalates in all toys and childcare articles [10]. The aim of the present article is to present an overview of the reproductive and developmental toxicity of phthalates in experimental animals as well as to discuss the relevance of these data for humans.

2 Effects on male reproductive system

2.1 Postnatal exposure

Reproductive adverse effects following postnatal exposure of males to phthalates are relatively well known. Exposure of adult male rats to high doses of certain phthalates results in rapid and severe changes in the testis [11–14]. The first finding of phthalate-induced testicular injury in experimental animals was reported by Shaffer *et al.* in 1945 [15]. The testicular effects are characterized by decreased testis weight and atrophy of seminiferous tubules with a progressive degeneration of germ cells, mostly spermatocytes and spermatids, which ultimately slough off into the tubular lumen [11, 12]. Tubular atrophy is accompanied by germ cell apoptosis, decreased testicular zinc levels and increased urinary zinc excretion [11, 14, 16, 17]. However, the alterations in spermatogenesis observed after exposure to reproductive toxic phthalates-like DEHP are thought to result from dysfunctions in Sertoli cells, which cannot adequately provide physical and metabolic support to germ cells [1]. Other studies have shown that interference of phthalates with follicle-stimulating hormone action on Sertoli cells is one likely component of testicular toxicity [18, 19]. Moreover, there is also experimental evidence showing that phthalates can target the Leydig cells and induce multiple hormonal disturbances [20, 21].

Toxic responses following phthalate exposure are largely influenced by age, with pre-pubertal and pubertal animals being generally more vulnerable than the adult counterparts. Usually, lower doses and shorter duration of exposures are needed to induce testicular damage in young rats [13, 22]. The reason for this age-dependent variation in testicular response to phthalates is not completely understood, but may involve differences in both tissue sensitivity and chemical disposition [1, 22].

Another important aspect of phthalate toxicity is the marked structure-activity relationship for the induction of reproductive effects. The testicular toxicity of these compounds depends in part on the length of the alcohol moiety (side chain) of the ester molecule [2]. In general, phthalates with medium- (*e.g.* dibutyl phthalate [DBP]) or branched long-side chains (*e.g.* DEHP) induce degenerative testicular lesions while those with short- (diethyl phthalate (DEP)) or linear long-side chains (di-*n*-octyl phthalate) are inactive [19, 23, 24]. However, most reproductive effects are not exerted by phthalate diesters themselves, but rather by their active primary monoester metabolites, which are considered the proximate toxicants [12, 14, 16]. In addition, recent experimental results indicate that secondary (oxidized) monoester metabolites may also contribute to adverse effects in males [25].

Although most reproductive effects have been described in rats, phthalates can induce testicular injury in several other species including mice [23, 24], guinea pigs [26] and ferrets [27]. However, some species, such as hamsters and non-human primates, seem to be less sensitive than rats [26, 28–31]. Part of this variability may be attributed to differences in phthalate bioavailability [28, 32]. For instance, following oral exposure in rodents, a significant amount of DEHP is absorbed on the form of mono-(2-ethylhexyl) phthalate (MEHP), because of rapid hydrolysis by gut lipases. However, the conversion of phthalate diesters into the active monoester metabolites in the gut seems to be less efficient in primates than in rats [1, 33]. In a study by Kurata *et al.* [29], no changes in testis weight or histopathology were observed in adult marmosets administered orally at 100, 500 or 2500 mg DEHP/kg/day for 13 wk. More recently, absence of testicular effects was also reported in young adult cynomolgus monkeys and juvenile marmosets exposed to high DEHP doses (500–2500 mg/kg/day) [30, 31]. However, non-human primates have not been extensively evaluated during fetal and neonatal periods, which represent developmental windows especially susceptible to exogenous insults. A recent work by Hallmark *et al.* [34] indicates that neonatal marmosets treated orally with 500 mg/kg/day DBP may respond similarly to rats in relation to changes in testosterone production and Leydig cell alterations, although marmosets seem to be able to reverse the suppression of testosterone production more efficiently than rats. Due to the lack of data on *in utero* and early postnatal exposures, it is not possible to draw conclusions regarding possible developmental effects in non-human primates.

2.2 Effects on male reproductive development

Recent toxicity studies indicate that exposure to certain phthalates results in severe disorders on the developing male rat reproductive system. Reproductive toxicity induced during the perinatal period was reported for DEHP [35–37], DBP [38, 39], butyl benzyl phthalate (BBP) [40] and to a lesser extent for the phthalate mixture diisononyl phthalate [35]. Male offspring rats exposed *in utero* or *in utero* and during lactation to high phthalate doses (*e.g.* 750 mg DEHP/kg/day or 500 mg DBP/kg/day) display reproductive tract abnormalities compatible with disruption of androgen-dependent development and impaired testicular function [35–38, 40, 41]. The phenotypic alterations manifested in male offspring include cryptorchidism, hypospadias (ectopic opening of the urethra), atrophy or agenesis of sex accessory organs, testicular injury, reduced daily sperm production, delayed preputial separation, permanent retention of nipples and decreased (feminized) anogenital distance.

Unlike other antiandrogens, which act by binding to the androgen receptor, phthalates disrupt the development of androgen-dependent structures mainly by inhibiting the fetal testicular testosterone biosynthesis [42, 43]. This effect is mediated by changes in gene expression of enzymes and proteins involved in testosterone production by fetal Leydig cells [44, 45]. Recently, the expression of another product of the fetal Leydig cell, the insulin-like factor 3 (insl 3), has been shown to be reduced in animals exposed to DEHP, DBP and BBP [46, 47]. Such effect might explain the incidence of abdominal cryptorchidism following exposure to these compounds, as insl 3 is involved in the initial stages of testicular descent into the scrotum [46].

Testicular lesions are seen early in the fetal testis with the presence of dysgenetic areas characterized by malformed seminiferous cords containing multinucleated gonocytes and aggregates of Leydig cells in the interstitial space [48]. In adult offspring, affected testes display reduced germ cell differentiation, Sertoli cell-only tubules, Leydig cell aggregates and multinucleated giant germ cells [35, 37, 48]. Figure 1 shows the presence of atrophic seminiferous tubules in the testis of an adult rat exposed *in utero* and during lactation to 405 mg DEHP/kg/day. Reductions in Sertoli cell number and/or proliferation have also been reported in neonatal rats treated with phthalates [13, 49]. However, this appears to be a transient effect as no changes in the number of Sertoli cells are observed later in life [13, 37].

Interestingly, the spectrum of effects obtained following perinatal exposure of rats to reproductive toxic phthalates shares many features with common human reproductive disorders, which collectively comprise the so-called testicular dysgenesis syndrome (TDS) [50]. According to Foster [47], the effects induced by phthalates in rats constitute a continuum of response with the most severe manifestations and highest incidence of reproductive tract malformations observed at high doses (*e.g.* 750 mg DEHP/kg/day). However, when the range of doses are extended toward lower exposures (*e.g.* from 5 mg DEHP/kg/day upward) we still observe some reproductive tract defects (although at much lower incidences) as well as the manifestation of other subtle alterations such as reduced sperm production [36, 51]. Similarly to the disorders induced by phthalates, the clinical expression of the TDS symptoms in humans may vary with the severity of the syndrome. Accordingly, less severe manifestations would result in impaired spermatogenesis while other symptoms like cryptorchidism, hypospadias and testicular cancer may be present in more severely affected individuals. A recent work by Swan *et al.* [52] provided the first evidence of an association between reproductive effects in human infants and exposure to phthalates. In a regression analysis, the authors described a negative relationship between concentration of four phthalate metabolites (monoethyl phthalate, mono-*n*-butyl phthalate (MBP), monobenzyl phthalate and monoisobutyl phthalate in maternal urine and the anogenital index (weight-adjusted anogenital distance) in male infants. The DEHP monoester MEHP was unrelated to anogenital index; however, the DEHP oxidative metabolites, mono-2-ethyl-5-oxohexyl phthalate and mono-2-ethyl-5-hydroxyhexyl phthalate were of borderline significance. Despite these results, additional evidence is needed to demonstrate a link between phthalate exposure and human disease.

2.3 Mode of action

In both adult and developing males, disturbance of Leydig and Sertoli cell functions constitutes an integral change for induction of phthalate effects. Recently, Lahousse *et al.* [53] presented results indicating that similar genetic targets are altered following fetal and pre-pubertal exposure to DEHP and DBP. Moreover, the structure-activity relationship for developmental reproductive toxicity of phthalates resembles that seen in adult and pubertal rats [35]. However, hormonal and local cell signaling perturbations may irreversibly alter reproductive and endocrine functions in a manner that may not be predicted from adult exposure.

It is important to note that most hormonal alterations associated with exposure to active phthalates, such as DEHP and DBP appear to be secondary to maldevelopment (dysgenesis) of the testis [54]. Sertoli and Leydig cells are active components in the fetal testis orchestrating the development of the reproductive tract and the testis itself [55]. Considering the existence of paracrine relationships

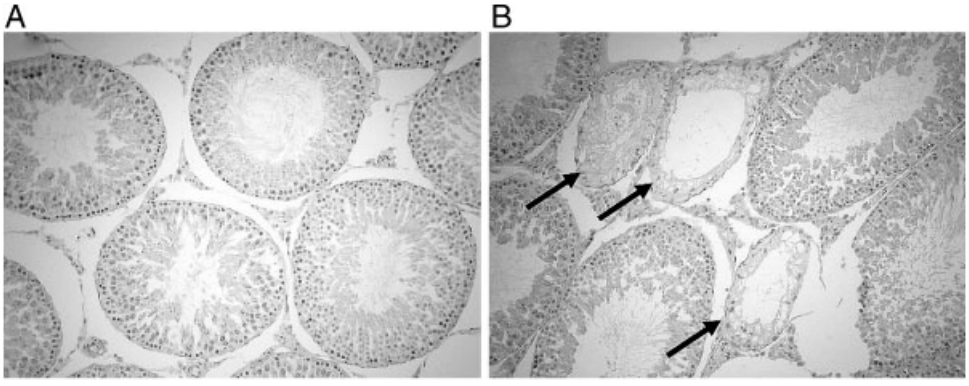

Figure 1. Testicular sections of adult rat offspring exposed *in utero* and during lactation to vehicle (A) or 405 mg DEHP/kg/day (B). DEHP exposed testis displays atrophic tubules (arrows) characterized by severe reduction of germ cell differentiation. Magnification 200×. Adapted from Andrade *et al.* [37].

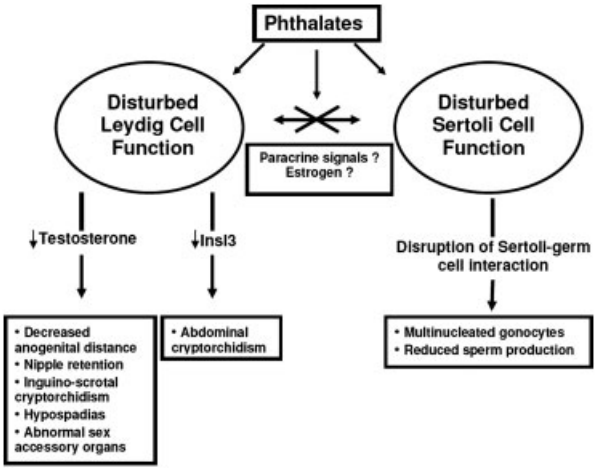

Figure 2. Diagrammatic representation of the cellular targets of phthalates in the fetal testis and the associated downstream hormonal and phenotypic alterations. Figure modified from Sharpe [55].

between the two cell types, phthalate-induced malfunction of Sertoli cells is likely to affect the physiology of neighboring Leydig cells and *vice versa*. However, it is uncertain whether the primary changes occur in one specific cell type or even in both types simultaneously. Figure 2 shows a schematic representation of cellular targets of phthalates in the fetal testis and the associated downstream hormonal and phenotypic alterations. However, although several target genes involved in the development and function of fetal Leydig and Sertoli cells have been identified so far [44, 45], the mechanism by which phthalates alter the expression of these genes are currently unknown.

A possible involvement of peroxisome proliferator-activated receptors (PPARs) has been suggested, as there is evidence that PPARs can alter gene expression of enzymes involved in testosterone biosynthesis and can also interact with or down-regulate other nuclear receptors that play roles in testis development [56, 57]. However, testicular toxicity induced by phthalates has been shown to be at least partially independent from PPAR activation, as suggested by results with PPAR-α knockout mice [57]. In addition, phthalates are unique among peroxisome proliferators with respect to the profile of testicular and reproductive alterations produced.

3 Female reproductive effects

3.1 General

In contrast to males, it is generally thought that the female reproductive system is much less sensitive to phthalates. However, recent evidence suggests that phthalates can also induce adverse responses in females following pre and postnatal exposure [58–60]. Initial studies demonstrated that the ovary is a target site for DEHP. Davis *et al.* [61] reported that a high DEHP dose (2000 mg/kg/day) results in prolonged estrous cycles, reduced serum estradiol levels and absence of ovulation in adult rats.

Fertility studies with crossover mating have shown that active phthalates-like DEHP and DBP can decrease the fertility of rats and mice through male and female-mediated effects [23, 62]. Recently, Gray *et al.* [60] reported that oral administration of DBP to female Long Evans rats from weaning through puberty, mating and gestation disrupts pregnancy maintenance at doses of 500 and 1000 mg/kg/day. In this study, DBP-induced midgestation abortions, which were associated with increased progesterone and decrease estradiol production. Teratogenic effects of phthalates have also been reported but the incidence of fetal malformations depends on dose, species and route of administration tested [2]. In general, most skeletal and visceral malformations are observed in mice after oral exposure [63, 64]. In rats, there is little indication of teratogenicity [2, 64].

Postnatal consequences of *in utero* and lactational DEHP exposure in rats were recently reported [58, 59]. Grande *et al.* [59] showed that DEHP exposure results in a delay in the age of puberty onset (vaginal opening) in female offspring at doses of 15, 45, 135 and 405 mg/kg/day. Interestingly, when male littermates were evaluated for preputial separation, a marker of puberty onset in male rats, a significant delay was observed at the same doses causing delayed vaginal opening in females [36] (Fig. 3). However, during adulthood, female offspring exposed *in utero* and during lactation did not present any sign of disturbed reproductive function, with the exception of an increase in the incidence of tertiary atretic follicles in animals exposed to the highest dose tested (405 mg/kg/day) [59]. No adverse changes were detected in estrous cyclicity, serum estradiol and progesterone concentrations or reproductive organ weights. This is in contrast with the results obtained with adult male offspring, which showed impaired testicular function and reproductive tract abnormalities at doses as low as 15 and 5 mg DEHP/kg/day, respectively [37]. Taken together, these results indicate that although changes were seen in both young male and female offspring exposed *in utero* and during lactation to similar doses, adult female offspring appear to be less sensitive to persistent effects on the reproductive system than their adult male counterparts.

3.2 Mode of action

The existing experimental data with females indicate that the ovary is a target organ for phthalates. Suppression of estradiol production was considered the primary functional effect in adult females [61, 65]. *In vitro* experiments by Davis *et al.* [65] showed that MEHP, the main DEHP metabolite, decreases estradiol biosynthesis in cultured rat granulosa cells by a mechanism involving suppression of aromatase enzyme activity. More recently, Lowekamp and Davis [66]

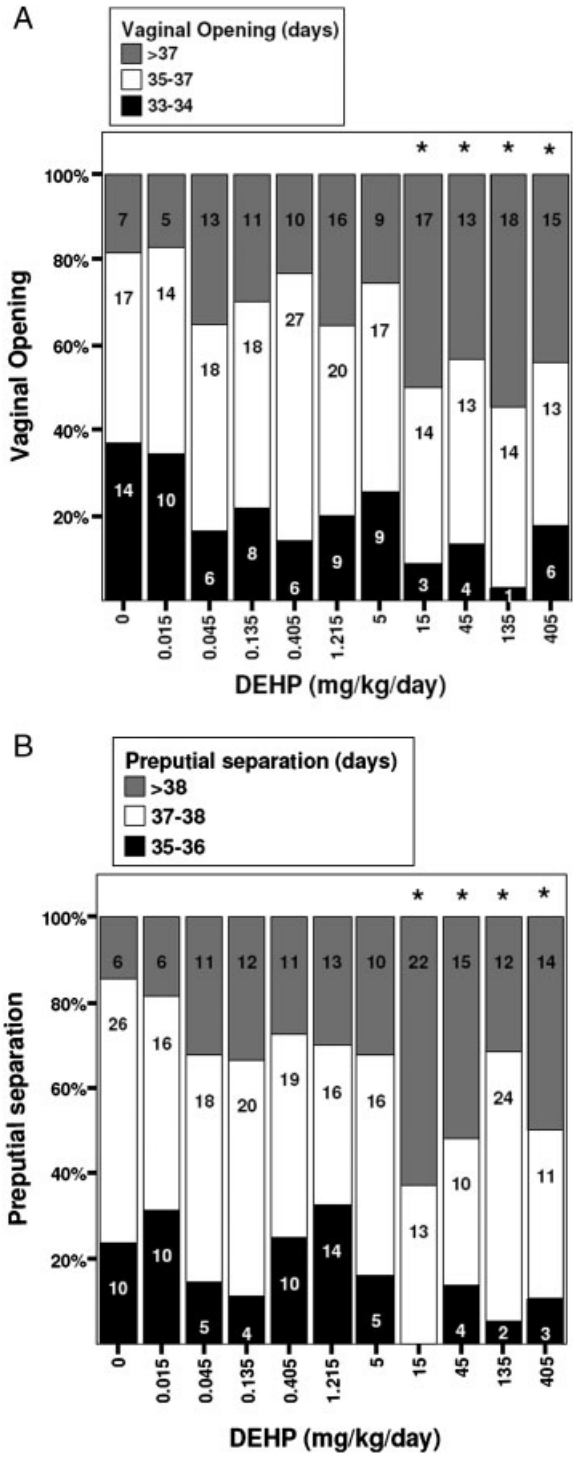

Figure 3. Vaginal opening (A) and preputial separation (B) in offspring rats exposed *in utero* and during lactation to a wide range of DEHP doses. Bars indicate the percentage of animals displaying vaginal opening or preputial separation in the assigned categories. Number of animals is indicated inside bars. Asterisk statistically different from control $p<0.05$ (Chi-Square). Figure adapted from Andrade *et al.* [36] and Grande *et al.* [58].

demonstrated that MEHP affects aromatase in cultured granulosa cells by decreasing aromatase transcript and, subsequently, protein levels. In addition, *in vivo* studies have shown that pre and postnatal exposure to high DEHP doses may result in altered number of ovarian follicles [59, 67].

As in the case of males, it has been proposed that activation of PPARs may be involved in some aspects of female reproductive toxicity. Lovekamp-Swan *et al.* [68] showed a molecular mechanism by which MEHP, through activation of PPAR-α and PPAR-δ, alters the expression of genes critical in granulosa cell estradiol production and metabolism. In addition, it has been hypothesized that MEHP may activate PPAR-δ, disrupting the timing of growth and differentiation of the ovarian follicles [69].

4 Low-dose studies and relevance of reproductive effects of phthalates to humans

The main question involving phthalates is whether the level of human exposure is sufficient to adversely impact male and/or female reproductive health. For most phthalates, the range of doses typically used in animal studies is three to four orders of magnitude greater than the estimated daily exposure of humans. However, recent low-dose studies indicate that some phthalates can induce biological changes in experimental animals at doses within the range of common human exposure [44, 70, 71].

Treatment of rat dams with active phthalates may result in non-monotonic (biphasic) dose-responses for the activity/expression of enzymes and proteins involved in the biosynthesis of steroid hormones in the offspring [44, 53, 70]. In addition, non-monotonic dose-responses and low-dose effects have been reported for endpoints not related to reproductive function [71]. In a recent study with DEHP, Andrade *et al.* [70] have shown a striking biphasic dose-response for aromatase enzyme activity in the brain of neonatal male rats, with low-dose inhibition and high-dose stimulation (Fig. 4). Since aromatase is a key enzyme in the biosynthesis of estrogens, phthalate exposure might be associated with disturbances of the normal balance between androgens and estrogens. Lehmann *et al.* [44] studied alterations in genes coding for steroidogenic enzymes in the fetal testis of rats exposed to DBP. In this study, the authors reported reductions in several genes at doses that approach maximum human exposure levels. However, it is important to note that the significance of such low-dose changes is largely unknown and that they cannot be taken as clear evidence for concern about human health. The current no observed adverse effect level adopted by the European Food Safety Authority [51] for DEHP is 5 mg/kg/day, which is based on a multigeneration continuous breeding study that identified alterations in male reproductive organs in F1 and F2 generations at an estimated dose (LOAEL) of 14 mg/kg/day [72] (http://ntp.niehs.nih.gov). The derived

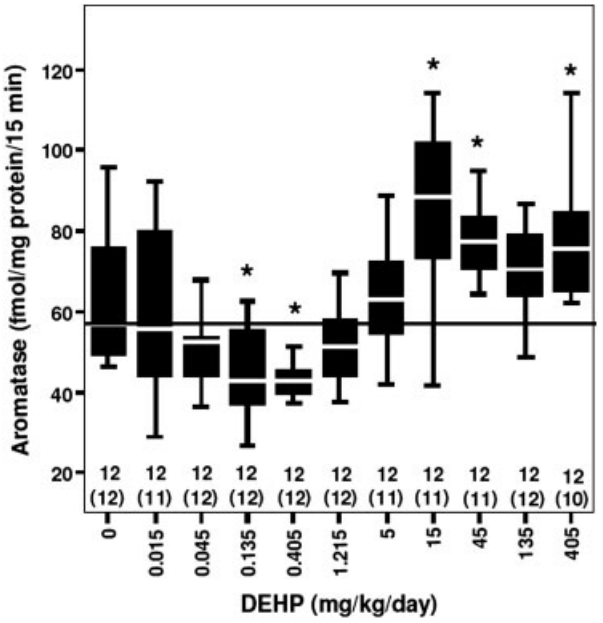

Figure 4. Non-monotonic dose-response profile for male rat brain aromatase activity following *in utero* exposure to DEHP. Aromatase activity was measured in brain fragments containing the hypothalamic pre-optic area of male pups on postnatal day 1. The number of pups is indicated below each box plot and the number of litters is in parenthesis. Box plots indicate medians, 25 and 75% quartiles, maximum and minimum values. The horizontal black line is the median of control group. Asterisk significantly different from control group ($p<0.05$). Figure adapted from Andrade *et al.* [70].

tolerable daily intake of 0.05 mg DEHP/kg/day is still well above the estimated median levels for human exposure, *e.g.* 0.0024 mg DEHP/kg/day for the general German population [73]. However, significantly higher DEHP exposures can occur in critically ill patients undergoing medical treatment, including neonates in intensive care units, as a result of DEHP leaching from medical devices.

In addition, the possibility of cumulative effects resulting from multiple exposures has raised additional concerns, as reproductive toxic phthalates can cause a similar spectrum of effects and molecular changes in rats [35, 53]. Howdeshell *et al.* [74, 75] demonstrated that DEHP, DBP, BBP, di-pentyl phthalate and diisobutyl phthalate could act in a cumulative, dose additive manner to reduce the testicular testosterone production by the rat fetal testis. More recently, Martino-Andrade *et al.* [76] showed that in addition to cumulative effects on testosterone production, maternal coadministration of DEHP and DBP is able to increase the diameter of seminiferous cords and induce gonocyte multinucleation in the rat fetal testis at doses that individually produce no significant changes in these variables.

Another important issue regarding the relevance of reproductive effects of active phthalates to human health is the existence of species differences in relation to both bioavailability and mode of action. As mentioned before, the rate of MEHP absorption in primates seems to be much slower than in rodents, as a result of lower levels of gut lipases necessary for the conversion of DEHP into its active monoester metabolite [1]. Few studies have addressed the relevance of phthalate effects in developing primates. In newborn marmosets, a single dose of 500 mg/kg/day of MBP, the active metabolite of DBP, suppressed blood testosterone levels 5 h after administration [34]. In this same study, attempts to demonstrate changes in testosterone production *in vitro* have failed in both rat and human fetal testis explants cultured with MBP. Therefore, the lack of *in vitro* effects in a responsive species as the rat precludes any reasonable conclusion on possible effects of DBP/MBP in the human testis [34]. In another study [77], there was no effect on basal or LH-stimulated testosterone production in human testes exposed *in vitro* to MEHP. However, MEHP significantly reduced the number of germ cells by increasing apoptosis. This is in accordance with the results published by Gaido *et al.* [78], showing that mice, which are resistant to phthalate-induced testosterone suppression, also display some typical testicular changes (*e.g.* gonocyte multinucleation) after DBP exposure.

On the other hand, results from recent epidemiological studies indicate associations of phthalate exposure with a number of human reproductive disorders including poor semen quality in adult males [79–81], short pregnancy duration [82], endometriosis [83, 84], pre-mature breast development in girls [85] and reduced anogenital distance in male infants [52]. However, most of these studies have limitations that do not allow us to establish clear conclusions on human health risks [86]. In addition, it is likely that no single factor is the cause of reproductive abnormalities such as those seen in the human TDS. In fact, high human phthalate exposures may be associated with other factors or lifestyle practices that are the actual causative agents [87]. For instance, some studies in human populations have suggested associations between monoethyl phthalate, a metabolite of DEP, and low-sperm quality [79–81]. However, DEP belongs to the group of phthalates that cause no reproductive effects either in adult or developing laboratory animals [86]. Additional studies using larger human populations are needed, before we can determine the real risks of phthalates to human reproductive health.

5 Concluding remarks

Phthalate esters are ubiquitous environmental contaminants that in general display low-toxicity. The reproductive effects induced by exposure of experimental animals to these compounds are relatively well characterized. In general, high doses are required to adversely affect the male and female reproductive system in adults, with the testis and ovary being considered the main target organs. However, the main concern involving phthalates is related to the effects induced in males after perinatal exposure. Recent

studies have shown that exposure to certain phthalates results in profound and irreversible changes on developing male reproductive system.

Although most reproductive tract abnormalities induced by phthalates occur at doses well above the estimated intake of the general population, the resemblance of phthalate effects with common human male reproductive disorders has raised concerns of a possible link between phthalate exposure and human disease. Moreover, recent experimental results indicate that biological changes can also be induced at low, human relevant doses and that different active phthalates can have cumulative effects. However, uncertainties in the epidemiological data base, difficulties in animal to human extrapolations and the lack of knowledge on the significance of low-dose effects for human health preclude a better understanding of the real risks for humans. Below, we present some of the main issues that should be addressed in order to clarify the implications of phthalates to human reproductive health:

(i) Epidemiological studies using larger human populations, including follow-up studies of infants exposed to high phthalate doses during medical treatment.
(ii) Investigation of phthalate effects in non-human primates during pre and early postnatal periods.
(iii) Advancements in mechanistic research and determination of the relevance of the mechanisms of action to humans.
(iv) Studies on the biological significance of the reported low-dose changes in the expression/activity of steroidogenic enzymes in experimental animals exposed to certain phthalates.
(v) Further investigation on possible cumulative effects of various active phthalates.

The authors have declared no conflict of interest.

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