Review

The effects of the phthalate DiNP on reproduction[†]

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Abstract

Di-isononyl phthalate (DiNP) is a high molecular weight, general purpose, plasticizer used primarily in the manufacture of polymers and consumer products. It can be metabolized rapidly and does not bioaccumulate. The primary metabolite of DiNP is monoisononyl-phthalate (MiNP) and the secondary metabolites include three oxidative derivatives of DiNP, which have been identified mainly in urine: mono-oxoisononyl phthalate (MOINP or oxo-MiNP), mono-carboxyisooctyl phthalate (MCIOP, MCOP or cx-MiNP), and mono-hydroxyisononyl phthalate (MHINP or OH-MiNP). The secondary metabolites are very sensitive biomarkers of DiNP exposure while primary metabolites are not. As the usage of DiNP worldwide increases, studies evaluating its potential reproductive toxicity are becoming more prevalent in the literature. In studies on female animals, the researchers found that the exposure to DiNP appears to induce negative effects on ovarian function and fertility in animal models. Whether or not DiNP has direct effects on the uterus is still controversial, and the effects on human reproduction require much more research. Studies on males indicate that DiNP exposure has disruptive effects on male reproduction and fertility. Occupational studies also indicate that the exposure to DiNP might induce negative effects on male reproduction, but larger cohort studies are needed to confirm this. This review presents an overview of the literature regarding the reproductive effects of exposure to DiNP.

Summary sentence

The phthalate DiNP has negative effects on gonadal function and fertility in both males and females.

Key words: phthalate, reproductive toxicity, DiNP, metabolite, testis, ovary.

Introduction

Phthalates are widely used compounds that humans are frequently exposed to. The different chemicals can be grouped into two types according to the number of carbon atoms or their molecular weight, short- and long-chain or low- and high-molecular weight. Different applications, classifications and toxicological properties are found between the two types. Di-isononyl phthalate (DiNP), a long-chain (high-molecular weight) phthalate, is increasingly being used to

replace di(2-ethylhexyl) phthalate (DEHP) (the other globally used long-chain phthalate) as the major plasticizer in flexible polyvinyl chloride (PVC) products [1, 2]. DiNP is considered to be a useful substitute for DEHP, since its primary and secondary metabolites are less toxic than those of DEHP [3].

DiNP can be used in different types of goods such as building products (including vinyl flooring coverings and wall coverings), food packaging materials, automotive applications, containers,

Figure 1. Schematic diagram of DiNP exposure, metabolism, excretion and biomonitoring. DiNP is absorbed by the gastrointestinal system where it is hydrolyzed to MiNP quite rapidly, or by inhalation or by dermal contact. It is mostly metabolized in the liver, and further oxidized into the secondary metabolites, oxo-MiNP, cx-MiNP and OH-MiNP. These metabolites are mainly excreted in the urine, which can be used to monitor exposure. Other body fluids, such as blood, amniotic fluid, breast milk and cord blood can also be used for biomonitoring in conditional circumstances. Since blood cannot be used as an excretion method but can be used for biomonitoring, we indicated this with the dashed line box. For bile, since it is a method for excretion, but cannot be used for biomonitoring, we used a light gray color.

electrical wires and cables [4, 5]. During recent years, investigators found that medical devices also appear to leach DiNP [6, 7]. Studies have reported that DiNP exposure can cause chronic toxicity and carcinogenicity. DiNP can induce liver injury and tumors in mice and rats [8] and peroxisome proliferator-activated receptor alpha (PPAR α) is thought to play a critical role in the effects of DiNP exposure during the process of hepatocarcinogenesis [9–11]. DiNP has also been reported to induce allergic airway inflammation [12], asthma [13–17], hypertension [18], increased risk of osteopenia [19], neuroinflammation in the brain [20] and brain injury [21–23], cognitive deficits and anxiety [24], aggravated allergic contact dermatitis [25, 26] and autoimmune thyroid disease [27]. Many of the negative impacts of DiNP exposure reflect increases in pro-inflammatory responses. Now, with the increased use of DiNP, more studies evaluating its potential role as a toxicant in male and female reproduction are being carried out. This review summarizes the literature regarding the reproductive effects of exposure to DiNP from the databases of PubMed, Scopus, and Web of Science published until August 1, 2020. We conducted a single broad literature search on DiNP and then performed title/abstract or full text screen for all outcomes.

Metabolism and biomonitoring of DiNP

Studies investigating the absorption, metabolism and excretion of DiNP carried out in rats have shown that after oral administration, the DiNP was being absorbed via the gastrointestinal (GI) tract and hydrolyzed to its monoester mono-isononyl-phthalate (MiNP) quite rapidly, while absorption via skin seems to be minor (<5%) [28, 29]. Subsequent further oxidation of MiNP to its secondary metabolites then occurs in the liver [30]. The secondary metabolites include three oxidative forms that have mainly been identified in urine: mono-oxoisonyl phthalate (MOINP or oxo-MiNP),

mono-carboxyisooctyl phthalate (MCIOP, MCOP or cx-MiNP), and mono-hydroxyisooctyl phthalate (MHINP or OH-MiNP) [31, 32] (as shown in Figure 1). They are metabolized and excreted rapidly and present no bioaccumulative effects. There are few published studies concerning the metabolism of DiNP focused on the female and male reproductive organs. One recent study reported that enzymes associated with phthalate metabolism are presented in the ovary and in isolated ovarian follicle culture systems, which indicate the mouse ovaries and follicles at different stages possessed variable metabolic capacity [33]. McKee et al. [29] observed that DiNP absorbed through oral or dermal exposure can be found in the testes. Although the percentage of the applied dose recovered in the testis was very low, ranging from 0 to 0.002% at day 7 after exposure, DiNP did reach the male reproductive organs and may have adverse effects if it reaches a high enough concentration, particularly with continuous exposure.

In humans, one study involving a single volunteer to monitor DiNP metabolism found a bi-phasic elimination pattern for each metabolite [34]. The elimination half-lives ($t_{1/2}$) for primary and secondary metabolites were 3 and 5 h separately in the first phase, and 12 and 18 h in the second phase, which begins approximately 24 h post-dosing. MiNP elimination was almost complete by the end of 24 h. Both OH- and oxo-MiNP were eliminated completely after 48 h post-dosing, but not the cx-MiNP. Even 48 h later, cx-MiNP still could be detected. In total, 43.6% of the ingested DiNP was eliminated via the urine within 48 h after dosing, indicating that other metabolites might exist. Another study that included 20 adult human volunteers, administered a much lower dose than that used by Koch et al. previously, showed similar excretion kinetics for DiNP [35].

Metabolites of DiNP can be detected in biological fluids, such as urine, blood and amniotic fluid. Several studies suggest that [36] urine metabolites (cx-MiNP, oxo-MiNP and OH-MiNP) are more

accurate biomarkers for assessment of DiNP exposure in rats [30, 37] and human [34, 38] than MiNP. Other human biological fluids, such as blood serum [39–41] and amniotic fluid [39] have also been used to monitor the exposure to DiNP. However, the levels of metabolites in the amniotic fluid were generally lower when compared to the concentrations in fetal plasma, and the correlation between the levels of secondary metabolites of DiNP in serum and in urine were only weakly to moderately positive. Thus, the authors suggested that researchers should avoid using amniotic fluid and serum samples for biomonitoring human exposure except for conditions when urine samples were not available, such as historical cohort studies. In addition, using only a spot urine analysis for assessment of personal long-term exposure is not very accurate because of the rapid clearance of DiNP metabolites. Hsu et al. [42] therefore, proposed to measure DiNP metabolites in rat hair and suggested to use these measurements as a new, non-invasive method for long-term monitoring of DiNP exposures.

Human biomonitoring (HBM) assessment values (e.g. values of specific phthalate metabolites in urine) developed by the German HBM Commission and Biomonitoring Equivalents (BEs) presented by Summit Toxicology, have been used for the exposure risk assessment of DiNP [43]. The detailed information of BEs for DiNP can be found in the Hays data [43, 44]. Daily intake (DI, $\mu\text{g/kg/day}$) estimates were calculated from the DiNP urinary biomonitoring data (from 1988 to 2010). In general, DI estimates of DiNP range from 1 to 2 $\mu\text{g/kg/day}$; the health-based exposure guidance values, range from 120 to 290 $\mu\text{g/kg/day}$, two orders of magnitude higher than the general DI value [45]. And according to recent studies, younger children had higher concentration of urinary phthalate metabolite levels than older children or adults [30, 46].

In summary, the secondary metabolites of DiNP present in the urine are good, representative biomarkers for assessment of human exposure to DiNP. Using the monoester metabolite MiNP as a biomarker seems to underestimate exposure to DiNP. Serum and amniotic fluid can also be analyzed if urine samples are not available. In addition, hair samples should be considered as an alternative for monitoring long-term exposure.

DiNP and effects on female fertility

Estrogenic activity of DiNP

Phthalates have been reported to mimic or antagonize the biological activity of several steroid hormones. A recombinant yeast screen was used to assess the estrogenic activity of DiNP. Using this *in vitro* screen, Harris et al. [47] found that DiNP displayed extremely weak estrogenic activity. Compared with other phthalates, DiNP showed the weakest estrogenic potency. The relative potencies in ascending order were DiNP < diethyl phthalate (DEP) < diisobutyl phthalate (DIBP) < dibutyl phthalate (DBP) < butyl benzyl phthalate (BBP). Estrogenic potencies ranged from approximately 1×10^6 to 5×10^7 times less than that of 17 β -estradiol. Using the XenoScreen YES/YAS screen system, Czernych et al. [48] also presented similar results with no significant estrogenic properties of DiNP. Another recent study from Sedha et al. [49] indicates that the phthalate compounds DIBP and DiNP have no estrogenic potential *in vivo*. Female rats, 20 days old, were orally dosed with DiNP (276 and 1380 mg/kg) once daily for 3 or 20 days and the potential estrogenic activity was assessed utilizing uterotrophic and pubertal bioassays. Neither dose of DiNP promoted vaginal opening or induced increase in uterine weight. In summary, none of the studies demonstrated any obvious estrogenic activity for DiNP.

Animal studies

Pre- and perinatal exposure and female reproductive effects. The potential developmental and reproductive toxicity of DiNP was evaluated in the Sprague–Dawley rat, using a series of doses (ranging from 0 to 1000 mg/kg/day) given to the rats from gestation days (GD) 6 through 15 [50]. Fertility parameters, such as corpora lutea number, implantation sites, pregnancy loss, and viable fetuses, were not different between the treatment groups. However, the incidence of fetal skeletal malformations increased at the highest dose tested (1000 mg/kg/day), as well as maternal toxicity effects (decreased maternal body weight and mean food consumption). Based on these findings, the authors proposed a conservative NOAEL (no-observed-adverse-effect-level), of 500 mg/kg/day for both maternal and fetal developmental effects.

The exposure period in the previous study ranged from GD 6 to GD 15, which did not include the critical period (beginning from embryonic days 16 to 18) for brain sexual differentiation [51]. Therefore Gray et al. [52] exposed rats to 750 mg/kg/day from GD 14 to postnatal day (PND) 3. Their study confirmed the previous results with no reduction in litter sizes or obvious maternal toxicity. However, they reported that both DEHP and DiNP treatment led to reduced pregnancy weight gain at GD 21 by 24 and 14 g, respectively. These results indicate that DEHP exposure affected pregnancy in female rats more severely than did DiNP.

A subsequent study in which pregnant, female Sprague–Dawley rats were fed a diet containing DiNP at 400, 4000 and 20 000 ppm from GD 15 to PND 10, showed that treatment at 20 000 ppm (equivalent of 1200–2600 mg/kg/day) resulted in a decrease in absolute ovarian and uterine weights but not the relative weights (relative to body weight; mg/100 mg body weight) in the F1 offspring prior to the onset of puberty [53]. At PND 21 and postnatal week (PNW) 11, the organ weights of both the ovary and uterus in the F1 offspring showed no significant differences among treated and untreated groups. There was a decrease in corpora lutea number/mm² in the ovaries at PNW 11, but the change was minimal. Similar to the results of Masutomi et al., Boberg et al. [54] found that the F1 female offspring that were exposed to DiNP (doses ranging from 0 to 900 mg/kg/day) from GD 7 to PND 17, had no significant differences in ovarian or uterine weight between treated groups when evaluated at PND 90. Research by Lee et al. [55] reported similar results. They fed pregnant Wistar–Imamichi rats with a soy-free diet containing 0, 40, 400, 4000, or 20 000 ppm of DiNP from GD 15 to PND 21. Thereafter, all offspring animals were administered a normal diet. The F1 offspring were analyzed in both PNW 8–9 and 19–20. Their results showed that estrous cycles were normal for all the treatment groups. There were also no differences in serum levels of LH, FSH, and E2 between the different treatment groups at PNW 20.

Taken together, these studies suggest that neither pre- nor perinatal exposure to DiNP have any significant effects on female reproductive tract organs but more in-depth functional analyses are needed.

Adult exposure and female reproductive effects. The potential reproductive toxicity of adult exposure to DiNP has also been investigated in multi-generational reproductive toxicity studies. Parental generation (P1) rats were fed with diets containing 0 to 1.5% DiNP. The offspring generation (F1) was fed a reduced ratio of DiNP as follows: 0, 0.2, 0.4, and 0.8%, because obvious toxic effects at the ratio of 1 and 1.5% were observed in the parental generation, including reduced maternal and offspring body weight, as well as

reduced offspring survival. Both generations were fed the DiNP-containing diets for 10 weeks prior to mating, and then remained on the diets throughout pregnancy and until offspring's PND 21. Observations and measurements were obtained at the beginning of DiNP treatment and continued through offspring's PND 21. From these experiments, the authors concluded that the classic reproductive parameters did not differ between the treatment groups. The overall NOAELs calculated for these effects mentioned above were approximately 500 mg/kg/day in the multi-generation study (0.8% in chow) and 1000 mg/kg/day (1.5% in chow) in the single-generation study [56].

The effects of short-term adult exposure to DiNP have also been investigated. Chiang et al. [57] used 39–40 days old female CD-1 mice and dosed them orally with a concentration range of 20 µg/kg/day to 200 mg/kg/day DiNP for 10 days. They reported that this 10-day exposure to DiNP during adulthood not only had short-term but also long-term consequences for the reproductive life of these mice. Short-term exposure to DiNP altered the distribution of ovarian follicle populations and the levels of sex steroid hormones (including E2, P4, T and FSH, but not inhibit (B) at multiple time points (immediately post-dosing, 3, 6 and 9 months post-dosing) [58]. In addition, the authors found that DiNP exposure during early adulthood led to late-life (12, 15, and 18 months post-dosing) negative consequences. In addition to alterations in ovarian follicle populations, hormone levels and estrous cyclicity, DiNP also decreased fertility, increased pregnancy loss and altered the sex ratio of pups (increased number of males) [58].

Previous studies have reported that pre- and perinatal exposures to DiNP have little to no effect on the uterus, and also that DiNP appears to have no estrogenic activity [49]. However, Hwang et al. [19] found that exposure to DiNP (2, 20, and 200 mg/kg i.p.) in intact C3H/HeN mice (8 weeks old) for 6 weeks, caused a reduction in the uterine weight compared to those of the control group, although the uterine weight was higher in intact than in ovariectomized females. Furthermore, hematoxylin and eosin staining of the uterus revealed that DiNP-induced atrophic-like alterations in the uterine epithelium and cell layers. These contradictory results from different studies may be due to: (1) different animal models (Sprague–Dawley rats, CD®(SD) IGS rats, C3H/HeN mice); (2) the timing of the exposure period (prenatal/perinatal versus adult); (3) the length of time of exposure (from 3 days to two generations); and (4) different dose ranges utilized. Overall, adult exposure to DiNP seems to be more harmful to female reproductive potential than pre- or perinatal exposure.

In addition to rodent models, Santangeli et al. [59] found that in adult zebrafish, exposure to DiNP (ranging from 0 to 4200 µg/L) for a period of 21 days not only adversely affected oocyte growth and maturation, but also disrupted gonadal development and induced poor reproduction. They also reported that expression of the autophagy gene *ambra1a* was significantly increased in oocytes exposed to DiNP at the concentration of 0.42 µg/L, but no changes were detected for apoptosis associated genes. This suggests that autophagy may play an important role during oocyte growth and death. In contrast, Patyna et al. [60] utilized the Japanese medaka (Japanese rice fish) as an animal model for multigenerational exposure studies and found no difference in egg production in the F0 or F1 generations among the treatment groups (1 µg/g fish/day). These differing results may be attributed to the different timings of treatments and differences in levels of exposure to DiNP.

Human studies

There are currently very limited data from human studies about the effects of exposure to DiNP in females. One study [61], consisting of a pool of 938 pregnant women from Greenland, Poland and Ukraine, found that the fecundability ratios (FR, indicates the probability of getting pregnant within a certain time period) were unrelated to DiNP exposure in these women. Time to pregnancy (TTP) is another parameter used to evaluate a woman's fecundity. Analysis of first-time pregnant women from the Greenland cohort determined that as serum levels of DiNP increased, the women experienced a longer TTP. This result suggests that greater exposure to DiNP may slightly reduce fecundity in women.

In summary, exposure to DiNP appears to induce negative effects on ovarian function and fertility in animal models, specially if exposure occurs during adulthood (summarized in Table 1). Whether or not DiNP has direct effects on the uterus is still controversial, and the effects on human reproduction require much more research.

DiNP and effects on male fertility

Androgenic and anti-androgenic activity of DiNP

The androgen agonist and antagonist activities of DiNP were assessed in a recent study. Using a yeast Xenoscreen YES/YAS assay (based on a β -galactosidase gene expression system), Czernych et al. [48] reported that phthalates (including DiNP) did not show any androgenic activity, since no significant stimulation of β -galactosidase gene expression was detected. The EC50 for 5 α -dihydrotestosterone was 5.15×10^{-3} M while for DiNP it was 0.110 M, which means that DiNP was 100 times less effective in inducing activation of androgen receptors (indicated by the synthesis of β -galactosidase) than 5 α -dihydrotestosterone. In contrast, a significant anti-androgenic activity was observed for all detected phthalates. Of the phthalates tested, DiNP had the strongest androgen inhibitory properties (IC50 = 0.0679 µM). Since DiNP is metabolized quickly when it enters the body, more studies are needed to evaluate the androgenic or anti-androgenic activity of the DiNP metabolites.

Animal studies

Prenatal exposure and male reproductive effects. The male reproductive system is a vulnerable target for phthalates, particularly during the development phase. Whether or not DiNP has negative effects on the male reproductive system is still somewhat controversial, but several studies have identified negative effects on male reproduction.

Steroidogenesis. Most studies indicate that an inverse relationship exists between DiNP exposure levels and testis testosterone (T) concentrations or expression of steroidogenic enzymes (as shown in Table 2).

Clewell et al. [39] reported that the T concentration in the testes of male fetuses was reduced when pregnant mice were dosed with DiNP (250 and 750 mg/kg/day) on GDs 12–19. Another study, using higher doses of DiNP (500 mg/kg/day or greater), also observed a decrease in fetal testicular T production in a dose-dependent manner after an exposure period from GD 14 to GD 18. The levels of steroidogenic enzymes, including StAR and Cyp11a were reduced as well [62]. Studies have shown that DiNP is less potent (2.3-fold) in reducing fetal testicular T production than other types of phthalates such as DEHP, DIBP, and DIHP [52, 62]. Li et al. reported that in utero exposure to DiNP at the concentration of 10 mg/kg/day from

Table 1. The effects of DiNP on female reproduction in animal studies.

Reference	Species	Doses	Exposure time	Main effects
Waterman et al. [50]	SD rat	0, 100, 500, and 1000 mg/kg/day	GD 6–15	No differences in fertility were found
Gray et al. [52]	SD rat	750 mg/kg/day	GD 14 to PND 3	No overt maternal toxicity or reduced litter sizes
Masutomi et al. [53]	SD rat	400, 4000, and 20 000 ppm	GD 15 to PND 10	No significant differences in ovary and uterus in the F1 offspring at PND 21 and PNW 11. A decrease in corpora lutea number/mm ² in the ovary at PNW11
Borch et al. [64]	SD rat	750 mg/kg/day	GD 7–21	No significant difference on the ovary and uterus weight between treated groups when evaluated at PND 90
Li et al. [63]	SD rat	10, 100, 500, and 1000 mg/kg/d	GD 12–21	F1 offspring in both PNW 8–9 and 19–20 had regular estrous cycles. No difference were found in serum levels of LH, FSH, and E2 in PNW 20
Waterman et al. [56]	SD rat	Dose 1: in the diet at doses of 0, 0.5, 1, and 1.5%; Dose 2: in diet at doses of 0, 0.2, 0.4 and 0.8%.	–10 weeks to GD 21 (Dose 1) PND 0–21 (Dose 2) 10 days	No change in any of the classic reproductive parameters: mating, female fertility, fecundity, gestational index, or length of gestation
Chiang et al. [57, 84]	CD-1 mouse	20, 100 µg/kg/day; 20, 200 mg/kg/day		Disrupted estrous cyclicity, increased pregnancy loss, decreased fertility, altered the sex ratio of pups, altered ovarian follicle populations, and disrupted hormone levels
Hwang et al. [19]	C3H/HeN mouse	2, 20, and 200 mg/kg/d	6 weeks	Reduced uterine weight, reduced thickness of the uterine epithelium and the number of cell layers
Santangeli et al. [59]	Fish	0, 0.42, 4.2, 42, 420, 4200 mg/L	21 days	Affected oocytes growth and maturation, leading to abnormal gonadal development and reproduction
Patyna et al. [60]	Fish	1 mg/g fish/day	Multigeneration	No difference in egg production in F0 and F1 generations

Footnote: GD, gestational day; ED, embryonic day; PND, post natal day; PNW, post natal week.

GD 12–21 inhibited insulin-like factor 3 (*Inlf3*) and *3β-HSD* gene expression and protein levels in fetal Leydig cells, while DiNP at 1000 mg/kg reduced testicular T levels at GD 21.5 [63]. Borch et al. found that at GD 21, DiNP exposure (750 mg/kg/day) from GD 7–21, reduced testicular T production ex vivo and T levels in testes and plasma of male fetuses, but increased plasma LH levels [64]. Another similar study also observed a decreased level of T in the testes of fetal mice on GD 21 when treated at the dose of 600 mg DiNP/kg/day, yet no alterations of plasma LH and T were found between the treatment groups [54].

In contrast, a study by Adamsson et al. showed no effect of fetal exposure to DiNP on expression of steroidogenic enzymes and production of T in the testis. Since the time window from embryonic day (ED) 13.5–17.5 is critically important for reproductive tract masculinization in the rat, Adamsson et al. [65] exposed pregnant Sprague–Dawley rat to DiNP (250 and 750 mg/kg) during this time period. They reported that the exposure to DiNP did not down-regulate key components of the steroidogenic pathway in the ED 19.5 male fetuses. Both testicular and adrenal protein levels of androgen receptor (AR), *3β*-hydroxysteroid dehydrogenase (*3β*-HSD), cytochrome P450 side chain cleavage (P450_{sc}, Cyp11a) and steroidogenic acute regulatory protein (StAR) showed no significant changes in male fetuses exposed to DiNP. Intratesticular T levels also did not show any change.

Studies utilizing exposure to phthalate mixtures that included DiNP in utero (GD 10.5 to birth, CD-1 mice) resulted in reduced T levels in male offspring at the age of 12 months, and decreased mRNA levels of *StAR*, *Cyp11*, and *Cyp17* [66]. These results were consistent with a study by Hannas et al. who used a mixture of nine phthalates (including DiNP) and observed similar results [62]. Although these studies identified negative effects of phthalate exposure on male fertility, one cannot be certain whether these effects would result from individual exposure to DiNP or whether these are cumulative effects from exposure to all the phthalates present in the mixture. Finally, research studies on the adult gilthead sea bream also determined that steroidogenesis was affected with the levels of T being significantly increased after exposure to DiNP for 21 days [67]. These differences in the relationship between DiNP exposure and T levels may be attributed to the different detection time points after dosing, different test methods, and different species (rat, mouse, fish) utilized in studies.

Morphological changes and defects in reproductive tissues Exposure to DiNP in utero can also cause morphological changes. Exposure to DiNP at 10 mg/kg/day from GD 12 to 21 caused an increase in Leydig cell size at GD 21.5. Leydig cell aggregation and multinucleated gonocytes also increased in a dose-dependent manner, with the lowest observed adverse-effect level (LOAEL) at 10 and 100 mg/kg respectively [63]. Multinucleated germ cells were also observed and increased in a dose-dependent manner in the fetal testes of rats (exposed from GD 12 to 19 to 250 and 750 mg/kg/day) when harvested at 24 h after final administration [39]. Similar results were also found in a study done by Boberg et al. [54]. In one study, male offspring mice that experienced prenatal exposure (GD 10.5 to birth) to a phthalate mixture (including DiNP) showed decreased gonadal, seminal vesicle, and prostate weights, and disrupted spermatogenesis at 12 months of age [66]. In contrast, other investigators reported that an even higher exposure concentration of 1000 mg/kg did not induce changes in testis volumes (measured at GD 20.5) [63]. These differences might be due to differences in the time points examined, exposure dose

Table 2. The effects of DiNP on male reproduction in animal studies.

Reference	Species	Doses	Exposure time/assessment time	Main effects
Borch et al. [64]	SD rat	750 mg/kg/day	GD 7–21/GD 21	Reduced testicular T production ex vivo and T levels in testes and plasma of male fetuses
Adamsson et al. [65]	SD rat	250 and 750 mg/kg/day	ED 13.5–17.5/ED 19.5	No effects on steroidogenic enzymes and intratesticular T level. Normal histology of fetal-type Leydig cells
Hannas et al. [62]	SD rat	500, 750, 1000, and 1500 mg/kg/day	GD 14–18/GD 18	Reduced fetal testicular T production in a dose-responsive manner, reduced <i>SlAR</i> and <i>Cyp11a</i> expression
Clewell et al. [39]	SD rat	50, 250, and 750 mg/kg/day	GD 12–19/GD 19	No change in the AGD at any of the tested doses. Reduced T concentration and increased multinucleated germ cells in the fetal testes at groups of 250 and 750 mg DiNP/kg/day
Li et al. [63]	SD rat	10, 100, 500, and 1000 mg/kg/day	GD 12–21/GD 21.5	A dose-dependent increase in fetal Leydig cell aggregation, multinucleated gonocyte, increased fetal Leydig cell size, decreased expression of <i>Ini3</i> and <i>3β-HSD</i> . Decreased testicular T levels at 1000 mg/kg dose
Barakat et al. [66]	CD-1 mouse	phthalate mixtures (20, 200 μ g/kg/day, 200, and 500 mg/kg/day)	GD 10.5–21/PND 21, PNM 2, 12	No effect on AGD. Decreased prostate, seminal vesicle and gonadal weights. Decreased serum T concentrations and mRNA expression of testicular <i>SlAR</i> , <i>Cyp11</i> , and <i>Cyp17</i> and impaired spermatogenesis at PNM 12
Gray et al. [52]	SD rat	750 mg/kg/day	GD 14 to PND 3/PND 13, PNM 3–7	Retained nipples/areolae, appeared testicular atrophy, epididymal agenesis, and hemorrhagic testis
Masutomi et al. [53]	SD rat	400, 4000, and 20 000 ppm	GD 15 to PND 10/PND 2, 27, PNW 8–12	Reduced testis weights before puberty and degeneration of meiotic spermatocytes and Sertoli cells in the testis in adulthood. No effect on AGD at PND 2
Lee et al. [55]	Wistar-Imamichi rat	40, 400, 4000, and 20 000 ppm	GD 15 to PND 21/PND 1, 7, PNW 8–9, 19–21	Decreased AGD at PND 1 while no serum T levels changes at PND 7. Decreased copulatory behavior, but no effects on LH and FSH when the male reached sexual maturity
Boberg et al. [54]	Wistar rat	300, 600, 750 or 900 mg/kg/day	GD 7 to PND 17/GD 21, PND 1, 13, 22, 200	Increased nipple retention, reduction in AGD, reduced sperm motility and increased sperm counts. No alteration in inhibin B (PND 22) and T (PND 90)
Clewell et al. [68]	SD rat	0, 760, 3800, or 11 400 ppm (target daily doses were 0, 50, 250, and 750 mg/kg/day)	GD 12 to PND 14/PND 2, 14, 49	Induced multinucleated germ cells at 3800 ppm and Leydig cell aggregates at 11 400 ppm on PND 2, reduced AGD at 11 400 ppm on PND 14. No alteration of T levels, AGD, nipple retention or reproductive tract malformations on PND 49
Waterman et al. [56]	SD rat	Dose 1: in the diet at doses of 0, 0.5, 1, and 1.5%; Dose 2: in diet at doses of 0, 0.2, 0.4 and 0.8%.	–10 weeks through mating to GD 21 (Dose 1) PND 0–21 (Dose 2)/GD 0–PND 21	No changes in mating, male fertility, and no testicular effects in either the P1 males, exposed as juveniles and young adults or the P2 (F1) offspring exposed in utero. No change in accessory sex organs in F1
Forner-Piquer et al. [67]	Gilthead sea bream	15, 1500 μ g/kg/day	21 days	Regressed testes and decreased percentage of motile sperm cells. Increased T level

Footnotes: GD, gestational day; ED, embryonic day; PND, post natal week; PNW, post natal month; PNM, post natal month. T, testosterone; *SlAR*, steroidogenic acute regulatory protein; *Cyp11a*, cytochrome P450 side chain cleavage; *Cyp 17*, Cytochrome P450 17 α -hydroxylase; C17,20-lyase; *Ini3*, insulin-like factor 3; *3 β -HSD*, 3 β -hydroxysteroid dehydrogenase; AGD, anogenital distance.

and chemical treatment (mixture including DiNP or just DiNP). Furthermore, prenatal exposure to DiNP did not show any changes in the anogenital distance (AGD) of mice when analyzed on GD 18–20.5 [39, 62, 63]. Finally, a study conducted in fish found that after 21 days of DiNP exposure at low dose (15 µg/kg/day) and high dose (1500 µg/kg/day), the low exposure group showed abnormal distribution of cell type within the testis, with the spermatogonia appearing as the predominant type. Sperm motility, the most reliable indicator of fertilization success in fish, was decreased [67].

In summary, prenatal exposure to DiNP at certain concentration ranges does exert negative effects on the male reproductive system. A dose of 10 mg/kg, or higher, leads to morphological changes while 250 mg/kg, or higher, causes functional changes. Thus, most studies have confirmed that DiNP has anti-androgenic effects in the male.

Peri- and postnatal exposure and male reproductive effects. During sexual differentiation, exposure to bioactive phthalates is thought to produce abnormal reproductive phenotypes and hormone levels in animals and humans. Several studies have demonstrated that perinatal and postnatal exposure to DiNP can induce reproductive steroid hormone alterations and malformations [52–55, 68].

Steroidogenesis Recent studies have indicated that exposure to DiNP for different time periods and doses resulted in no alterations in testes or serum T levels at PND 7, PND 49 or PND 90 [54, 55, 68] (as shown in Table 2). One study exposed rats to DiNP from GD 15 to the day of weaning (PND 21) using doses ranging from 40 to 20 000 ppm and observed no changes in serum T levels at PND 7. When these exposed rats reached sexual maturity, they displayed decreased copulatory behavior, but their serum levels of LH and FSH were not affected by the DiNP treatments [55]. Both Boberg et al. [54] and Clewell et al. [68] reported no alterations in testes T levels on PND 90 or on PND 49 separately after exposure to DiNP. Boberg et al. [54] also found that the inhibin B levels in male offspring at PND 22 showed no difference between DiNP-treated groups and the controls.

Morphological changes and defects in reproductive tissues Diverse malformations, including testicular malformations and epididymal agenesis, were found in offspring rats after exposure on GD 14–PND 3 in a study by Gray et al. [52]. Another study [53], which exposed maternal Sprague–Dawley rats to DiNP at 400, 4000, and 20 000 ppm from GD 15 to PND 10, showed that treatment with DiNP at the highest level resulted in reduced testis weight before puberty but not in adulthood and degeneration of meiotic spermatocytes and Sertoli cells in the testis in adulthood. A similar study conducted by Clewell et al. [68] demonstrated that DiNP exposure (from GD 12 to different times after birth)-induced multinucleated germ cells at 3800 ppm and Leydig cell aggregates at 11 400 ppm on PND 2. However, in this study, global endpoint analysis did not show any permanent reproductive tract defects (hypospadias, cleft phallus, epididymal agenesis, abnormal epididymis or testes) in rats collected on PND 49 with any treatment dose of DiNP. Boberg et al. [54] also found no evident morphological alterations in male reproductive organs (seminal vesicles, prostates and testes) at PND 90 after exposing animals to 300–900 mg/kg/day of DiNP from GD 7 to PND 17, but they observed that exposure to DiNP at 900 mg/kg caused reduced sperm motility and increased sperm counts.

In addition, peri- and postnatal exposure to DiNP can result in changes in anogenital distance (AGD), although this is still controversial. Some studies have observed that exposure to DiNP caused a reduction in AGD at 11 400 ppm on PND 14 but not on PND 49 [68], at 40–20 000 ppm on PND 1 [55] and at 900 mg/kg on PND 90 [54]. In contrast, no alteration in AGD was observed in DiNP treatment groups at 750 mg/kg [52] or at 400–20 000 ppm on PND 2 [53]. Both Gray et al. [52] and Boberg et al. [54] found that male rats exposed to DiNP (750 mg/kg/day or higher within GD 7–17 period in Gray's study and from GD 14 to PND 3 in Boberg's study) had retained nipples/areolae when examined at PND 13 or at 3–7 months of age. However, in Clewell et al.'s study, the nipple retention was not observed on PND 14 or 49 after exposing to DiNP (0–11 400 ppm, daily targeted dose range 0 to 750 mg/kg/day DiNP) from GD 12 to PND 14 [68].

Taken together, these peri- and postnatal treatment studies, with exposure periods ranging from GD 7 to PND 21, show some variations in results such as the effects on AGD and reproductive tract malformations. These different outcomes can be attributed to differences in timing of exposure, length of exposure, doses used and method of measurement. However, one can conclude that exposure to DiNP does cause some anti-androgenic effects on the male reproductive system.

Multi-generational exposure to DiNP and male reproductive effects. The potential multi-generational effects of DiNP on male reproduction and fertility have also been investigated. Waterman et al. [56] tested male mating ability, male fertility ratio, and testis and accessory sex organ weights in a multi-generation study. The detailed treatment information is mentioned above in adult exposure and female reproductive effects section. In brief, treatment of two generations with DiNP (P1: 0, 0.5, 1, and 1.5%; F1 (P2): 0, 0.2, 0.4, and 0.8%) resulted in no alterations in these classic reproductive indices in either of the two generations. Furthermore, no differences in the accessory sex organs differences were observed in the F1 generation. Based on their results, the authors suggest that the estimated no observed adverse effect levels (NOAEL) for testicular atrophy was 960 mg/kg/day for the juveniles and adults in one-generation study and 470 mg/kg/day for P1 juveniles and adults or P2 offspring (PND 0–21) in the two-generation study.

When considering all the available studies carried out in animal models (summarized in Table 2), it appears that exposure to DiNP has disruptive effects on male reproduction and fertility. Inverse associations have been found between DiNP exposure and testosterone levels, Leydig cell function and testis development, as well as semen parameters. DiNP reduced fetal testicular T levels in a dose-dependent manner at doses of 500 mg/kg bw DiNP or greater, induced reproductive malformations at 750 mg DiNP/kg/day or greater, reduced sperm motility, and led to retained nipples/areolae. The effects on AGD are less clear perhaps because of differences in the way the measurements were carried out between studies and the ages of the animals at the time of measurement.

Human studies

Prenatal exposure and male reproductive effects. Studies in rodents have shown that prenatal exposure to DiNP can lead to increased incidence of cryptorchidism and hypospadias, reduced testicular volume (TV), reduced T levels and induced smaller AGD in males. In humans, there are also several studies that have explored the relationship between DiNP exposure and male reproductive function (summarized in Table 3).

Table 3. The effects of DiNP on male reproduction in human studies.

Study	Design (n)	Sample	Observation object (age/parameters)	Main results
Bornehag et al. [69]	Mother-son pair (196)	Urine (G 9–11 w)	19–21 months/AGD	Inverse associations were found between AGD and DiNP metabolites
Jensen et al. [70]	Mother-son pair (273)	Urine (mean: G 28 w) (G 20.4–30.4)	3 months/AGD, penile width and weight	No dose–response association was found
Axelsson et al. [71]	Mother-son pair (112)	Serum (mean 12 w) (G 6–35 w)	18.37 ± 0.41 years/testicular size, semen quality and reproductive hormones	DiNP is negatively associated with reproductive function of adult men
Hart et al. [72]	General population (216)	Serum (G 18 and 34 w, pooled)	20 years/testicular volume, semen parameters and serum reproductive hormone concentrations	Negative correlations between the levels of antenatal DiNP metabolites and testicular volume, a positive correlation with serum total T levels.
Jensen et al. [73]	Case (270 cryptorchidism, 75 hypospadias)/control (300)	Amniotic fluid G 15.6 weeks	Steroid hormones (T and androstenedione), <i>INLEF3</i>	Increased odds ratio but not significantly
Mieritz et al. [74]	Cross-sectional study (555)	Urine samples	6.07–19.83 years/pubertal stages, T, the presence of pubertal gynaecomastia	No evidence of anti-androgenic effects of phthalates in these healthy boys
Joensen et al. [75]	Cross-sectional study (881)	Urine samples	19.5 ± 1.3 years/FSH, LH, E2, T, inhibin B, SHBG, semen quality	%MiNP negatively associated with the ratio of T/LH and T/FSH and positively associated with SHBG. No associations between hormone levels or semen quality and urinary concentrations of DiNP metabolites
Specht et al. [77]	Cross-sectional study (589)	Serum samples	>18 years/FSH, LH, E2, T, SHBG, FAI, semen quality	Inverse associations between serum levels of serum T and the DiNP metabolites. Negative associations between some metabolites and SHBG, semen volume and total sperm count
Woodward et al. [76]	Cross-sectional study (1420)	Urine samples	>20 years (median: 47)/total T, E2, SHBG and free T, bioavailable T, and FAI	Levels of DiNP metabolites were not statistically significant associated with sex hormone concentrations
Rana et al. [78]	Case–control study (152/75)	Serum	Infertile and fertile men/sperm count, motility and morphology	DiNP is more frequently detected in serum of infertile men than in fertile men. No significant correlation was observed between DiNP and semen quality parameters in the infertile males

Footnotes: AGD, anogenital distance, from the center of the anus to the posterior base of the scrotum. FAI, free androgen index; SHBG, sex hormone-binding globulin; *INLEF3*, insulin-like factor 3; T, testosterone; G, gestational; w, weeks; FSH, follicle stimulating hormone; LH, luteinizing hormone; E2, estradiol.

The AGD has been used as one measure to evaluate reproductive toxicity in males. Bornehag et al. [69], evaluating the AGD of 196 boys at the age of 19–21 months, reported the strongest and most significant inverse associations with the maternal urinary DiNP metabolites (including OH-MiNP, oxo-MiNP and the sum of DiNP metabolites) measured at first-trimester of pregnancy (9–11 weeks). In a similar study, the Odense Child Cohort study that included 273 mother-son pairs, Jensen et al. [70] had different findings. They found no dose–response association between AGD or penile width (measured at 3 month old boys) and maternal urinary phthalates metabolites (including MiNP, OH-MiNP, oxo-MiNP and cx-MiNP), evaluated at approximate gestational age of 28 weeks. These distinct results might be due to the low levels of phthalate exposure occurring in this population, the differences in time of sample collection and the various monitoring times of offspring in the two studies.

More studies have focused on the relationship between prenatal phthalate exposure and reproductive toxicity on the adult male offspring. One study with 112 mother-son pairs [71] found that sons (18.37 ± 0.41 years old) in the highest exposure tertile during gestation for a specific DiNP metabolite (maternal sera cx-MiNP from gestational week 6 to 35) had lower total testicular volume, reduced semen volume, and 30% higher levels of FSH when compared to the sons in the lowest exposure tertile group. A recent study by Hart et al. [72] reported that DiNP metabolites in maternal serum collected at 18 and 34 weeks of gestation had negative correlations with testicular volume of the adult offspring (about 20 years old), but a positive correlation with serum total T levels. These contradictory results might be attributed to the complex interactions among several hormonal axes including hypothalamus-pituitary-gonadal axis, growth factors and thyroid hormone, which also can be affected by DiNP exposure and then influence the level of T in humans.

Amniotic fluid, which is suitable for evaluating fetal environmental exposure, has been used to explore the correlation between phthalate exposure and abnormal male reproduction. Jensen et al. [73] analyzed the DiNP metabolite cx-MiNP levels in amniotic fluid samples from a Danish biobank (collected at second-trimester of pregnancy). Their results showed an increased chance of developing hypospadias and cryptorchidism when the cx-MiNP levels increased. As the samples used in this study were collected from patients between 1980 and 1996, when DiNP was not in widespread use, further studies with more recent patient populations are warranted.

Postnatal studies and male reproductive effects. Several studies have now reported on the associations between DiNP metabolite levels in boys/men and various reproductive parameters. A cross-sectional study organized by Mieritz et al. [74] recruited a large cohort of 555 healthy boys (ages 6.07–19.83 years) to investigate these relationships. Their analysis of boys' serum T levels, clinical characteristics and phthalate metabolites in morning urine (included DiNP metabolites: MiNP, OH-MiNP, oxo-MiNP and cx-MiNP) did not reveal any association between phthalate exposure and T levels, timing of puberty, or pubertal gynaecomastia. A potential limitation of the study is that the metabolite measurements were carried out on a single urine sample and phthalate metabolites are known to have short half-lives. Reliance on only a single time point for measurement may not accurately reflect the overall levels of these phthalate metabolites. Thus, study designs including more sampling are needed to provide a complete representation of metabolite levels and how these may correlate with reproductive function.

Joensen et al. [75] studied a cohort of 881 healthy Danish men, and reported that the free androgen index (FAI) was 15% lower [95% CI: $-0.23, -0.08$] in the highest MiNP quartile than in the lowest quartile ($P < 0.001$). Adjusted %MiNP (the ratio of MiNP to the total DiNP metabolites) had a negative association with the ratio of T/FSH or T/LH and positive association with sex hormone-binding globulin (SHBG). These results indicate a decreased production of T combined with an inhibition of the pituitary–hypothalamic axis in men with a high %MiNP. However, few associations were found between each individual metabolite of DiNP and reproductive hormone levels or semen quality in final regression models. A similar study was conducted by Woodward et al. [76] who analyzed a cohort of 1420 males. Levels of DiNP metabolites were not statistically significantly associated with sex hormone concentrations within this cohort of men.

A more recent cross-sectional study utilizing a cohort of 589 male partners (>18 years) of pregnant women recruited within 3 years (May 2002–Feb 2004) from Greenland, Poland and Ukraine produced opposite results [77]. The researchers measured the secondary metabolites of DiNP in serum as well as serum FSH, LH, E2, T, SHBG, FAI and semen quality. Negative associations were found between some DiNP metabolites and serum T, SHBG, total sperm count and semen volume. The differences in results between the studies above may be due to the different DiNP metabolites tested, as well as different samples collected, and the fact that the primary metabolite of DiNP is not considered an accurate exposure marker. Moreover, a study including fertile and infertile men found that DiNP is more frequently detected in serum of infertile men but no significant association was observed between DiNP and semen quality [78].

Overall, the results from these postnatal exposure studies suggest there may be a negative impact of DiNP on specific reproductive parameters in men (see in Table 3). However, as DiNP use and exposure are increasing, more distinct effects may be identified in further studies.

Occupational exposure and male reproductive effects. As mentioned previously, the daily intake dose of DiNP is about 1–2 $\mu\text{g/kg/day}$ for the general population. The recommended acceptable daily intake dose for DiNP ranges from 120 to 290 $\mu\text{g/kg/day}$. In addition, the European Chemicals Agency (ECHA) has set the Derived No-Effect Level (DNEL) for the general population as 0.075 mg/kg for oral and 0.35 mg/m³ for inhalation exposure, and the corresponding BE value for cx-MiNP was 250 $\mu\text{g/L}$ [79, 80]. However, occupational exposure standards for DiNP have not yet been established. One study found that workers manufacturing PVC materials have six to ten-fold higher exposure levels to DiNP than the adult general population. Their daily exposure reaches up to 26 $\mu\text{g/kg/day}$ [81]. Another study reported similar results, they found that plastisol workers had pre and post work-shift concentrations of DiNP metabolites that were approximately 5–10 times and 20-times higher than those of the general population exposure [82]. Henrotin et al. [83] reported that short-term (3 days) exposure to DiNP in occupational settings significantly increased DiNP metabolites in men's urine and they presented a decrease in total serum T. In addition, using a questionnaire for participants, they found that workers with direct DiNP exposure had more self-reported erectile problems than workers without direct exposure at the workstation. It is, therefore, necessary to consider the potential risk of this greater occupational exposure to DiNP on human health. A recent study recommended to use the P95 (95th percentile) value of cx-MiNP as a reference value for occupational

exposure, which was 32.3 µg/L [80]. However, this value should be used as a standard or to further calculate occupational exposure with caution, because this study included a very small number of workers (5–8 people) [80–83]. More biomonitoring studies with larger cohorts, including longer exposure situations are needed for proper risk evaluation [5].

Conclusion and future directions

DiNP production and use have increased over the past years, accompanied by increased human exposure risk resulting in rapidly increased levels of DiNP metabolites detected in human biological samples. Thus, evaluation of the potential hazards of such exposure on fertility are needed. Based on previous data, neither pre- nor perinatal exposure to DiNP has significant effects on female reproduction. However, adult exposure does have some negative effects on adult female reproductive parameters in a rodent model. Even a short-term DiNP exposure resulted in life-long consequences including altered ovarian follicle population and distribution, steroid hormone levels, disrupted estrous cyclicity, decreased fertility and altered sex ratio of pups. In contrast, in humans, whether or not a short-term exposure to DiNP would result in similar consequences requires further study. Based on limited information, we do know that a longer time to pregnancy (TTP) was observed in first time pregnant women with a higher serum level of DiNP. Many questions still need to be answered, such as, whether there is any relationship between exposure to DiNP and pregnancy loss. Does exposure to DiNP influence the length of pregnancy and/or increase the risk of preterm delivery? Does DiNP exposure influence the outcomes of IVF? Does exposure to DiNP increase the incidence of reproductive diseases such as polycystic ovary syndrome (PCOS) or endometrial cancer? Moreover, the precise mechanism of how DiNP works on these organs is also not clear and needs further exploration.

Research in male animals has demonstrated that DiNP has anti-androgenic effects. Human studies also indicate that exposure to DiNP may be associated with alterations in reproductive hormone levels and semen quality. To date, some consequences are still controversial, such as the effects on AGD. While the negative effects of DiNP exposure on males in animal models have been confirmed and shown to be dose dependent, effects in humans are still unclear. Further studies are needed to assess the effects of adult exposure to DiNP in men, especially occupational exposure. In addition, the molecular mechanisms by which DiNP acts on reproductive tissues in the male need to be determined.

Due to concerns regarding the potential human toxicity of DiNP, a new compound, 1,2-cyclohexane dicarboxylic acid, diisononyl ester (DINCH), is now being considered as a safer alternative to DEHP and DiNP. Compared to DEHP, DINCH is less toxic and considered a responsible substitute. However, in a recent study, Σ DINCH metabolites (sum of DINCH metabolites) was associated with lower total T levels in men aged ≥40 years [76], while no association was found between Σ DiNP (sum of DiNP metabolites) and any sex hormone concentrations. Further investigations will need to be carried out to explore more thoroughly the potential harmful effects of exposure to DINCH on reproductive functions and fertility in both females and males.

Author contributions

SY, RBA and RAN all contributed equally to the writing, editing and revising of this manuscript.

Conflict of Interest

The authors have declared that no conflict of interest exists.

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