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Effects of intravenous and oral di(2-ethylhexyl) phthalate (DEHP) and 20% Intralipid vehicle on neonatal rat testis, lung, liver, and kidney

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Abstract

The highest human exposures to the plasticizer di(2-ethylhexyl) phthalate (DEHP) occur through intravenous (iv) exposure from medical procedures. Rodent toxicity studies, mainly using oral exposures, have identified male reproductive toxicity after developmental exposure as the primary concern. Other organs are also affected by DEHP and route may influence the degree of target organ involvement. Cammack et al. (2003) reported a critical study focused on testicular toxicity using oral and iv exposures of neonatal Sprague-Dawley rats to 60, 300, or 600 mg/kg body weight/day DEHP in Intralipid vehicle. The present study followed the same dosing paradigm and included assessment of additional organs to evaluate the potential utility of this design for DEHP alternatives. Reduction of testis weight was observed in all DEHP treatment groups and germ and Sertoli cell toxicity was observed at the two highest doses with both routes. Lung granulomas occurred in all iv DEHP groups, possibly related to increased fat particle size in DEHP lipid emulsions. Lung alveolar development was inhibited after both oral and iv high dose DEHP. Toxicity of oral Intralipid vehicle was observed in germ and Sertoli cells. The lack of such effects after iv vehicle exposure suggested that this may be a gut-mediated effect.

Keywords

DEHP; reproductive toxicity; lung toxicity; Intralipid; developmental exposure

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

1. Introduction

DEHP [di(2-ethylhexyl) phthalate] has long been widely used as a plasticizer to add flexibility to a variety of polyvinylchloride (PVC) products, including medical devices (e.g. tubing, blood bags). While the major source of exposure to DEHP for the general population is through the diet, the highest exposures occur through essential medical procedures. The subpopulation with the highest exposure level is infants in neonatal intensive care units (NICU), where exposures are mainly intravenous (iv) and can approach levels that are associated with toxic effects in rodents (FDA/CDRH, 2001; Mallow and Fox, 2014; NTP, 2006; SCENIHR, 2015). The FDA has estimated that the aggregate exposure of a 4 kg infant in the NICU is on the order of 3 mg DEHP/kg body weight (bw)/day (FDA/CDRH, 2001). More recently, Mallow and Fox (2014) estimated exposure based on a critically ill 2 kg infant at approximately 16 mg DEHP/kg bw/day. Due to the well-studied toxicity of DEHP, particularly as a male reproductive toxicant in rodents, medical device manufacturers have reduced the use of DEHP as a plasticizer and several potential alternatives to DEHP have been introduced, although DEHP is still used in many devices and high levels of metabolites relative to those in the general population are still detected in the urine of NICU patients (Pinguet et al., 2019; Stroustrup et al., 2018a,b).

Most DEHP toxicity studies have used the oral route of exposure and studies using an intravenous route of exposure in young animals are rare. In addition, while a major focus of toxicological studies on DEHP exposure during development has focused on male reproductive toxicity, multiple other organs in rodents show toxic effects after exposure to DEHP. The extensively studied liver toxicity of DEHP has been largely attributed to a peroxisome proliferator-activated receptor alpha-mediated mechanism not relevant to humans (Corton et al., 2014; Rusyn et al., 2008), but the mechanisms responsible for toxicity in other organs have not been so clearly defined. For example, oral exposure of rats to high doses of DEHP during development can adversely impact lung maturation (Liang et al., 2018; Magliozzi et al., 2003; Rosicarelli and Stefanini, 2009), an effect of potentially high relevance to NICU patients, particularly if this occurs at lower exposure levels through a parenteral route. Cammack et al. (2003) conducted a study using emulsions of DEHP in 10% Intralipid at 60, 300, and 600 mg DEHP/kg bw/day intravenously and 300 and 600 mg DEHP/kg bw/day orally to neonatal rats for 21 consecutive days starting at postnatal day (PND) 3–5. Effects on the testis of the treated rats, including weight decreases, partial depletion of the germinal epithelium, and/or decrease in the diameter of the seminiferous tubules from both routes of exposure were reported at the two highest doses, with somewhat more severe effects from the oral route and an apparent NOAEL of 60 mg DEHP/kg body weight/day in the animals dosed intravenously. In the experiment described here, we have followed a slightly modified version of the protocol of Cammack et al. (2003), with the dosing of male pups starting at PND 3 for 5 and 21 consecutive days and with histological examination of the lung, kidney, and liver in addition to the testis at both ages. The purpose of the study was to replicate the earlier study, a critical study in regulatory safety assessments, with respect to testicular endpoints and to expand the investigation to other organs using this exposure paradigm to assist in determining whether age and route of exposure require additional attention in the evaluation of DEHP alternatives.

2. Materials and Methods

2.1 Chemicals

The purity of DEHP (CAS # 117-81-7; Sigma-Aldrich, St. Louis, MO; Aldrich Lot # 07627JD, 99.7% declared by manufacturer) was confirmed by gas chromatography (J&W DB-1 30m × 0.32mm capillary column, Agilent, Santa Clara, CA) with flame ionization detection (GC-FID). The vehicle for both intravenous (iv) and oral administration was Intralipid 20%, manufactured by Fresenius Kabe, Uppsala, Sweden and distributed by Baxter Healthcare Corporation, Deerfield, IL (Lot # 10BC3028).

2.2 Animals and experimental design

All animal work was approved by the NCTR Animal Care and Use Committee. Animals were housed in polycarbonate cages with hardwood chip bedding, wire lids, and microisolator tops. NIH-41 irradiated diet (Purina Mills, St. Louis, MO) and filtered tap water were provided ad libitum. The animal room was on a 12-h light/dark cycle and received 10–15 air changes per hour. Temperature in the animal room was maintained at $23 \pm 3^{\circ}\text{C}$ with a relative humidity of $50 \pm 20\%$. Date-mated female rats (Sprague-Dawley/CD23/NctrBR) were obtained from the NCTR colony and allowed to deliver their litters. Male pups from all litters born on a given day were randomly assigned before PND 3 (day of birth = PND 0) to treatment groups such that no litter mates were in the same treatment group and the mean treatment group weight was approximately equivalent. All pups in a given treatment group were housed with a single foster dam until the dam was removed at PND 21 at which time the pups remained together. Each pup was uniquely identified by a paw tattoo.

The study design used is a modification of that described by Cammack *et al.* (2003) and the dose levels are those used in that study. Beginning on PND 3, male pups were dosed daily by oral gavage or by tail iv injection with vehicle (Intralipid 20%), 60, 300 or 600 mg DEHP/kg bw in Intralipid 20%. While the low dose is higher than human exposure levels on a mg/kg basis, scaling the dose for body surface area (Reagan-Shaw *et al.*, 2008) results in a human equivalent dose of approximately 14 mg/kg bw/day, close to the NICU exposure estimated by Mallow and Fox (2014). Six pups were used per dose group, and each dose group was dosed for 5 or 21 consecutive days. For iv dosing, tail veins were dilated by application of isopropyl alcohol and were visualized with a magnifying glass with a light source. Injections were administered with 30–25-gauge half inch needles, depending on the size of the animals. For gavage dosing, a 30-gauge half inch needle with polyethylene tubing attached or stainless-steel oral gavage needles were used, depending on the size of the animal.

2.3 Dose preparation and lipid emulsion droplet size determination

DEHP formulations of 18, 90, and 180 mg/mL 20% Intralipid were placed in an ice bath and sonicated with a Sonics Vibra cell probe (#630–0419) for 5 minutes (repeated periods of 30 seconds on, 5 seconds off) at 40% amplitude to produce the dosing emulsions. The emulsion concentrations were determined by GC-FID to be $\pm 13\%$ of the target and stability was demonstrated for 3 weeks. The injection or gavage volume was $3.3 \mu\text{L/g}$ bw, based on the individual body weights collected just prior to dosing. Observation of lung granulomas at all

iv doses of DEHP prompted post hoc evaluation of the liposome size distribution in the vehicle and all DEHP dose levels using a Mastersizer 2000 equipped with a Hydro SM sample dispersion unit (Malvern Instruments, Malvern, UK).

2.4 Necropsy

After 5 and 21 consecutive days of dosing (PND 7 and 23, respectively), animals were euthanized approximately 3 hours post-dosing. Pups were anesthetized with CO₂ and anesthesia was confirmed by lack of response to a foot or tail pinch. Blood was collected by cardiac puncture, and death was ensured by cervical dislocation. Body weights were recorded, and liver, kidneys, lungs, and testes were removed, weighed, and further processed as described below. On PND 23, the brain, spleen, prostate, seminal vesicles, and epididymides were also removed and weighed. Paired organs were weighed together. Brain weight was collected primarily for use in normalizing organ weights as an alternative to body weight since brain weight is generally more stable when treatment affects body weight (Sellers et al., 2007).

2.5 Histopathology procedures

Depending on the age at necropsy, kidneys (PND 7 and 23), liver (PND 7 and 23), lungs (PND 7 and 23), spleen (PND 23), prostate (PND 23), epididymides (PND 23), and seminal vesicles (PND 23) were examined grossly, removed, and preserved in 10% neutral buffered formalin (NBF). Lungs were inflated with air and fixed in 10% NBF before being embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for histopathological analysis. Kidneys, liver, and lungs from the PND 23 rats were trimmed, processed, embedded in infiltrating media (Formula R, Leica Biosystems, Inc., Buffalo Grove, IL), sectioned at approximately 5 µm, and stained with H&E. The other tissues above were processed to block and held. In addition, the left testis from both PND 7 and 23 rats was fixed in modified Davidson's fluid for 48 hours; after fixation, the testis was sectioned transversely, and half was embedded in glycol methacrylate and the other half frozen in liquid nitrogen. The plastic-embedded testis was sectioned at 2 µm and stained with periodic acid–Schiff (PAS). A read-down of the PND 23 dose groups (high dose and control tissues evaluated first, other dose groups read only if adverse effects were apparent at the higher dose) to determine a NOEL was done for kidney, liver, lung, and testis. A read-down to determine a NOEL was done for all PND 7 tissues, where effects were observed at PND 23. When applicable, non-neoplastic lesions were graded for severity as 1 (minimal), 2 (mild), 3 (moderate), or 4 (marked).

2.5.1 Seminiferous Tubular Diameter and Seminiferous Epithelium Area—

Seminiferous tubular diameters and areas of the epithelium in the testes were measured for PND 23 rats. H&E stained sections of testes were scanned, and images were obtained by an Aperio Imaging System (Leica Biosystems, Buffalo Grove, IL). Diameters of 100 randomly chosen tubules were measured per animal (section) for each control and DEHP (60, 300, and 600 mg/kg bw/day)-treated rat. For consistency, the tubules were measured along the perimeter of a quadrangle drawn inside the testis section with corners touching the tunica. The diameters were measured using the Aperio Ruler Tool. Because the tubules were not perfectly circular, mostly due to the sectioning angle, the smaller diameter (width) was

chosen for the measurement. For seminiferous epithelium area measurements, sections of testes for each group were scanned (Aperio Imaging System) to make high resolution digital images. The cross-sectional area (mm²) of each testis, consisting mostly of germ cells contained within the seminiferous tubules, was calculated using the Aperio positive pixel count algorithm.

2.7 Statistical analysis

Continuous data were analyzed by a two-way ANOVA with dose and route as factors to assess potential interactions. Treatment groups were compared to controls by Dunnett's test within exposure arms and t-tests were used to compare oral and iv controls for each endpoint. Histopathology data were analyzed within exposure arms with an exact step-down Jonckheere-Terpstra test, which incorporates both incidence and severity scores and assumes a monotonic response. T-tests were used to compare the effects of dose on body and organ weights at the same DEHP dose level in the oral and iv dosing arms and the Holm method was used to adjust for multiple comparisons. An exact Kruskal-Wallis test was used to compare the effects of dose on histopathology endpoints at the same dose level in the oral and iv dosing arms and the Holm method was used to adjust for multiple comparisons.

3. Results

3.1 Body and organ weights after 5 and 21 consecutive doses

Body weight collected at the time of necropsy was significantly reduced relative to vehicle control in the oral 600 mg/kg bw/day dose group at PND 7 (Table 1) and in the oral and iv 600 mg/kg bw/day dose groups at PND 23 (Table 2).

Selected organ weights were collected at necropsy (Tables 1 and 2 for PND 7 and 23, respectively). Two-way ANOVA results (Supplemental Table 1) indicated a significant dose*route interaction for liver, both absolute and relative to body weight, and for absolute testis weight at PND 7 and for prostate and spleen weights at PND 23.

Liver and testis were affected at both ages. At PND 7, the mean liver weight relative to body weight was increased in the 300 and 600 mg/kg bw/day groups of both oral and iv exposures, while the absolute liver weight was increased in the iv 600 mg/kg bw/day group (Table 1). At PND 23, liver weight relative to body weight was significantly higher in the iv 300 and 600 mg/kg bw/day dose groups and in the oral 600 mg/kg bw/day dose group. The liver weight relative to brain weight was significantly higher in the iv 600 mg/kg bw/day dose group (Table 2).

At PND 7, testis weights were significantly affected only by oral dosing, with lower absolute weights in all dose groups. Mean testis weights relative to body weights were also significantly lower in the two highest oral dose groups. In the iv-dosing arm, the mean absolute testis weight in the 600 mg/kg bw/day dose group was 14% lower than the control, but this difference was not statistically significant. At PND 23, testis weights expressed as absolute, relative to body weight, and relative to brain weight were significantly reduced relative to controls in the 300 and 600 mg/kg bw/day dose groups of both exposure arms. In

the oral and iv 60 mg/kg bw/day dose groups, testis weights were lower than controls, but the differences were significant only for relative to body weight for the iv exposure and for absolute and relative to brain weight for the oral exposure (Table 2). In PND 23 animals, other male reproductive organs were also weighed. Absolute epididymis weight was decreased in the oral 600 mg/kg bw/day dose group, while seminal vesicle weights (absolute and relative to brain weight) were decreased in all orally dosed groups. Absolute prostate weight was decreased in the 600 mg/kg bw/day dose groups of both oral and iv exposures, and the mean prostate relative to brain weight was also decreased in the iv 600 mg/kg bw/day dose group.

At PND 7, kidney weight relative to body weight was significantly greater than the control in the iv 600 mg/kg bw/day dose group, while at PND 23 kidney weights (absolute and relative to brain weight) were affected only in the oral dosing arm at 300 and 600 mg/kg bw/day. Absolute lung weight was significantly lower in the oral 600 mg/kg bw/day dose group on PND 7, but there were no effects on lung weight at PND 23. Spleen weights (absolute and relative to brain weight) were significantly reduced in the oral 600 mg/kg bw/day group, while spleen weight relative to body weight was increased in the iv 600 mg/kg bw/day group.

In pairwise comparisons between iv and oral exposures at each dose level, there were no significant differences between body or any organ weights on PND 7. In similar comparisons at PND 23, the testis weights relative to body weight were significantly lower in the 300 and 600 mg/kg bw/day oral exposure groups. The vehicle control prostate weight relative to body weight was significantly lower in the oral exposure arm and the spleen weights (absolute and relative to body and brain weights) were significantly lower in the oral 600 mg/kg bw/day dose group.

3.2 Histopathology

Kidney, liver, lung, and testis were processed for histology. The results for testis and lung are presented below. The minimal effects observed in the kidney (degeneration of renal tubules, mineralization) and the expected effects, based on the literature, seen in the liver (hepatocyte hypertrophy, peroxisome proliferation, glycogen depletion) are presented in the Supplemental Material.

3.2.1 Testis, PND 7—There were no statistically significant differences between the testis lesions diagnosed in the iv and oral vehicle controls at PND 7 (Table 3). In the oral treatment groups, Sertoli cell apoptosis of minimal severity occurred in both the vehicle control (3 of 6) and 60 mg/kg bw/day (4 of 6) groups (Table 3, Supplemental Figure 1). In the 300 and 600 mg/kg bw/day groups, the proportions of affected animals were 5 of 5 and 5 of 6, respectively, with some animals showing higher severity. The high dose group differed significantly from the vehicle control. For the iv route of exposure, apoptotic Sertoli cells were observed only in the 600 mg/kg bw/day dose group.

Gonocytes were significantly affected in a dose-responsive manner by DEHP treatment, both iv and oral, with a greater number of atypical mitotic figures and multinucleated gonocytes (Table 3, Supplemental Figure 1). Only the high dose group showed increased mitotic

alteration in the iv-treatment arm, while mitotic alterations were increased in all three dose groups relative to the control group upon oral exposure (Table 3). The 300 and 600 mg/kg bw/day groups in both the iv and oral treatment arms had treatment-related increases in polynucleated gonocytes. Occasionally, apoptotic gonocytes were observed across dose groups (Table 3, Supplemental Figure 1).

Interstitial (Leydig) cell hyperplasia was also observed. Clusters or aggregates of a few fetal Leydig cells (4–6 cells) occurred in 50% of control and treatment groups alike. Clusters or aggregates of fetal Leydig cells were subjectively assessed for size comparing them to the subpopulation of smallest diameter seminiferous tubules in the histologic section. The only significant difference was an increase in the highest dose iv group (Table 3)

3.2.2 Testis, PND 23—In PND 23 animals, the major testicular effects included apoptosis of germ cells, depletion of germinal epithelium, and cytoplasmic vacuolization of Sertoli cells (Table 3, Supplemental Figure 2). The oral vehicle (20% Intralipid) control showed marked ongoing apoptosis of pachytene spermatocytes in the testis of 5 of 6 rats. Depletion of the germinal epithelium and cytoplasmic vacuolization of Sertoli cells were also observed in the oral, but not the iv, vehicle controls (Table 3, Figure 1).

In the 600 mg/kg bw/day group, and to a lesser extent in the 300 mg/kg bw/day group from both exposure routes, most of the apoptotic cells were leptotene and zygotene spermatocytes, as pachytene spermatocytes were largely absent, likely due to depletion by this time. A few apoptotic spermatogonia and pre-leptotene spermatocytes were also observed. For the oral exposure groups, the number of apoptotic germ cells in the seminiferous tubules decreased with increasing dose, as reflected in the lower severity scores. This likely resulted from the loss of increasing numbers of germ cells in the dosed groups by the time of sacrifice. In contrast, there was essentially no treatment effect on apoptotic germ cells in the iv groups, with low-grade apoptosis present in all groups, including the vehicle control. The severity of germ cell depletion increased in a dose-responsive manner in the 300 and 600 mg/kg bw/day groups in both the iv and oral exposure groups. Depletion was predominantly due to loss of primary spermatocytes, with pachytene losses greater than leptotene and zygotene. Sertoli cell vacuolization tended to correlate positively with germ cell loss (Table 3). Many of the larger vacuoles were associated with Sertoli cell apical cytoplasm. In addition to these larger vacuoles in the apical cytoplasm, there appeared to be an increase in fine vacuoles or cytoplasmic rarefaction adjacent to or surrounding Sertoli cell nuclei in all orally exposed groups, as well as the 300 and 600 mg/kg bw/day groups treated intravenously.

Morphometric data compiled for the seminiferous tubular diameter and testicular tissue area (mostly germ cells in the seminiferous tubules) correlated inversely with the diagnosed severity scores for germ cell depletion (Table 4). For both the iv and oral exposures, both endpoints were significantly different from the vehicle controls in the 300 and 600 mg/kg bw/day groups. The tubule diameter was also significantly lower than the vehicle control in the 60 mg/kg bw/day oral exposure group. The tubule diameter in the oral vehicle control animals was significantly lower than that in the iv vehicle control animals (Table 4).

3.2.3 Lung, PND 7 and PND 23—Lesions observed in the lungs of control and DEHP-treated animals are summarized in Table 5. Multifocal granulomas (Supplemental Figure 3) were observed in 94% of the iv-exposed PND 23 DEHP-dosed rats (60, 300, and 600 mg/kg bw/day combined), while none were observed in the iv vehicle control rats or any orally exposed animals (Table 5). This prompted further processing and examination of lung from the PND 7 rats treated by both routes of exposure. In the PND 7 pups, only the iv-exposed rats in the 300 and 600 mg/kg bw/day groups manifested lung granulomas of minimal to mild severity only. As reflected by the greater mean severity, there appeared to be more granulomas in the younger rats compared to PND 23 rats. The granulomas consisted of a central space containing sequestered wispy filamentous, amphophilic material in the alveolar wall, and surrounded by usually one or more layers of macrophages and lymphocytes, but occasionally, multinucleated giant cells were present (Supplemental Figure 3). The material sequestered at the core of the granulomas was not birefringent by polarized light microscopy, suggesting that it was not a crystalline or particulate material.

Multifocal hemorrhages were present only in the 600 mg/kg bw/day iv-exposed groups of PND 7 (4 of 6) and PND 23 (1 of 6) rats. Hemorrhage was diagnosed only if erythrophagocytosis was observed, indicating that the bleeding occurred before death, rather than as a likely result of lung lobe puncture during blood collection by cardiac puncture.

Moderate to marked interstitial inflammation (pneumonia) was found in the 600 mg/kg bw/day iv-exposed rats in both PND 7 (1 of 6) and PND 23 (2 of 6) animals. One of six PND 23 rats in the oral vehicle control group showed minimal inflammation. Increased numbers of histiocytes scavenging the alveolar spaces of lungs of a few rats in both iv- and oral-exposed PND 23 300 (iv, 1 of 6) and 600 (iv, 1 of 6; oral, 2 of 6) mg/kg bw/day dose groups, as well as the eosinophilic crystals present in lungs in 4 of 6 of the PND 7 600 mg/kg bw/day iv group rats, may also indicate DEHP treatment-induced lung damage (Table 5).

The lungs from the PND 23 600 mg/kg bw/day rats in both iv (5 of 6) and oral (4 of 6) exposure groups presented parenchymal development malformation, manifested by a subjective morphological evidence of fewer air spaces and apparent decreased alveolar septation compared to the control group (Table 5, Figure 2). This lesion was also diagnosed in the oral vehicle control group and in the oral and iv 60 and 300 mg/kg bw/day dose groups at lower incidence. Rigorous quantitative morphometry to evaluate the mean area of the airspaces and the number of airspaces per unit lung area was not possible since the inflation of the lungs at necropsy was not standardized. Assessment of PND 7 lung was not possible because nearly all the rats had iatrogenic hemorrhage and atelectasis caused by needle damage to the lung during blood harvest.

3.3 Assessment of emulsion droplet size in control and DEHP preparations

Given the lung histopathology results, particularly after iv exposure, the droplet size of the 20% Intralipid and DEHP emulsions was assessed post-hoc. The droplet size in the 20% Intralipid control emulsions was less than 1 μm in diameter, consistent with the approximate diameter of 0.5 μm stated in the package insert. As the DEHP concentration increased, an increasing volume percentage of droplets greater than 5 μm in diameter was observed

(Intralipid control, 0 %; 18 mg DEHP/mL, 1 %; 90 mg DEHP/mL, 7 %; 180 mg DEHP/mL, 20 %; Supplemental Figure 4).

4. Discussion

The toxicity of DEHP has been extensively studied, but there are few data on the toxicity of parenteral DEHP in developing animals, an exposure situation of concern due to the high human exposures to DEHP from medical procedures in the NICU. Koch et al. (2005) presented data from a single iv dose of DEHP to an adult human indicating that the extent and pattern of urinary metabolites excreted between 4 and 24 hours after dosing were comparable to those following a single oral dose. It was argued that differences in dose-response following the two routes were likely largely due to the developmental stage at the time of exposure rather than differences in exposure to active metabolites (Koch et al., 2005). However, parenteral dosing results in a transient internal exposure to unmetabolized DEHP that does not occur after oral dosing (Pollack et al., 1985). An early peak of DEHP in blood following iv treatment was confirmed in the present study (personal communication, N. Twaddle and D. Doerge, data not shown). The testicular toxicity of DEHP has largely been attributed to mono-(2-ethylhexyl) phthalate (MEHP) given that treatment with MEHP gives a similar pattern of toxicity at lower doses (Foster et al., 2001; Gray and Beamand, 1984; NTP, 2006). In vitro studies using various cell types have suggested that DEHP and MEHP can result in toxicity through different mechanisms (Takeuchi et al., 2005; Thomas et al., 1982; Wang et al., 2012; Wu et al., 2018), and iv administration of DEHP has been reported to result in lung toxicity not observed after oral dosing, although the dosing vehicle emulsion may play a large part in determining the extent of the lung effects (FDA/CDRH, 2001). Thus, continued evaluation of potential route-specific toxicity of DEHP seems warranted.

Our study utilized the exposure regimen reported in the study design of Cammack et al. (2003), which is the publicly released version of the study that served as the critical study in the 2001 FDA safety assessment of DEHP released from PVC medical devices (FDA/CDRH, 2001). While the Cammack et al. (2003) study reported only data on male reproductive toxicity and included a recovery group in which the toxicities reported were largely reversed, the present study included histological examination of additional tissues, included earlier ages for testicular examination of lesion development, and did not include a recovery group. An additional change was the use of 20% Intralipid as the vehicle due to the lack of commercial availability of the 10% Intralipid used as the vehicle by Cammack et al. (2003). This substitution may have had consequences in the oral dose arm that will be further discussed below.

The report of Cammack et al. (2003) focused on the testes and reported an iv NOAEL of 60 mg/kg bw/day, with reduced testis weights, partial depletion of the seminiferous epithelium and reduced tubule diameter observed at the 300 and 600 mg/kg bw/day dose levels. Oral dosing with 300 and 600 mg/kg bw/day resulted in similar, but generally more severe, effects than those observed with the iv route. No Sertoli cell lesions were reported at any dose level or dosing arm. The reduced testis weights persisted in iv-dosed recovery animals, but no microscopic lesions were noted. Minimal residual effects (focal decreased tubule

diameter in some animals) and decreased testis weights remained in the orally treated recovery animals. Our results obtained immediately after repeated exposures were generally similar to the results of Cammack et al. (2003), although significant reductions of absolute testis weight (PND 7 and PND 23, oral) or testis weights relative to brain (PND 23, oral) or relative to body weights (PND 23, iv) were observed at 60 mg/kg bw/day (Tables 1 and 2). In the 60 mg DEHP/kg bw/day oral dose group, seminiferous tubule diameter was also significantly reduced relative to control. In addition, treatment-related effects on Sertoli cells were observed in both PND 7 (apoptosis) and PND 23 (vacuolization) with both iv and oral exposures at the 300 and 600 mg/kg bw/day levels. Sertoli cells vacuolization upon phthalate treatment has been reported previously in rats exposed postnatally, with younger rats more susceptible than older rats (Chapin et al., 1988; Creasy et al., 1983; Dostal et al., 1988; Foster et al., 1982; Richburg and Boekelheide, 1996; Sjöberg et al., 1986). Dostal et al. (1988) reported that a 5-day oral DEHP treatment of rats beginning on day 6 of age resulted in a reduced number of Sertoli cells and found that recovery was evident in older animals. They did not observe degeneration of the Sertoli cells and hypothesized that the reduction in number was due to an inhibition of proliferation. Treatment of older animals, beyond the time when Sertoli cell proliferation ceases in the rats at PND 16–18 (Picut et al., 2018; Sharpe et al., 2003), did not result in lesions. Li et al. (2000) treated 3-day-old rats orally with a single dose of DEHP and observed multinucleated germ cells and a decrease in Sertoli cell proliferation, although they could not rule out a contribution of Sertoli cell death. Treatment of organ cultures from PND 3 rats with MEHP also resulted in decreased Sertoli cell proliferation (Li and Kim, 2003). Sjöberg et al. (1985) reported Sertoli cell vacuolization after repeated iv infusions of 500 mg/kg bw/day DEHP to rats starting on PND 25 or 40. Thus, the findings of Sertoli cell toxicity in the present study seem consistent with these previous studies, although such effects were not reported by Cammack et al. (2003). At PND 7, multinucleated gonocytes were also increased at the two highest dose levels in both the iv and oral dosed groups, consistent with what has been reported in other phthalate studies (Nardelli et al., 2017; Parks et al., 2000).

An unexpected observation was an adverse effect of the oral 20% Intralipid vehicle on the testes that was not observed in the iv controls. Intralipid 20% is a fat emulsion consisting of 20% soybean oil, 1.2% egg yolk phospholipids, 2.25% glycerin, and water that is commonly used for parenteral nutrition as a source for calories and essential fatty acids. The major component fatty acids in soybean oil, as stated on the 20% Intralipid package insert, are linoleic acid (44–65%), oleic acid (19–30%), palmitic acid (7–14%), linolenic acid (4–11%), and stearic acid (1.4–5.5%). In the PND 7 testis, apoptotic Sertoli cells were observed in the oral, but not iv, Intralipid controls. More striking effects were observed at PND 23, where marked germ cell apoptosis occurred in the oral controls, while only minimal germ cell apoptosis was observed in the iv controls. Rats in this early juvenile stage of development have been previously described to have some pachytene spermatocytes undergoing apoptosis, which has been attributed to limited testosterone production at this age (Jahnukainen et al., 2004; Picut et al., 2015). This is consistent with the observation in the iv controls, but not with the marked level of apoptosis in the oral controls. Furthermore, all the oral control animals showed depletion of the germinal epithelium and 83% of the oral controls had mild to moderate cytoplasmic vacuolization of the Sertoli cells. We are not

aware of rodent studies other than that of Cammack et al. (2003) that have used Intralipid as an oral vehicle. In that case, 10% Intralipid was used and there was no mention of adverse testicular effects in the oral versus iv Intralipid controls. To our knowledge, a similar dosing regimen over this same developmental period (PND 3- PND 23) with other lipid-rich vehicles has not been reported. The observation of an effect of a bolus of lipid administered orally, but not intravenously, is reminiscent of the incretin effect that results in the release of glucose-dependent insulintropic peptide (GIP) and glucagon-like peptide 1 (GLP-1) from enteroendocrine cells in the gut (Lindgren et al., 2011; Nauck and Meier, 2018; Vamvini et al., 2016). Tremellen et al. (2019) reported that oral, but not iv, Intralipid in adult men suppressed testosterone levels and hypothesized that this resulted from the release of GLP-1. Jeibmann et al. (2005) reported that GLP-1 reduced the pulsatile component of testosterone release without altering luteinizing hormone in an experiment involving nine adult male subjects. While testosterone was not measured in the present study, the effects observed on the testes, as well as the lower weight of the prostate in oral vehicle controls relative to iv vehicle controls could have resulted from reduced testosterone. The role of GLP-1 and reduced testosterone in the observed adverse effect in the testes of the young rats in the present study requires further evaluation. In adult mice, the GLP-1 receptor has been reported to be expressed in Sertoli cells, but not germ cells or testosterone-synthesizing Leydig cells, while a GLP-1 receptor agonist countered the adverse effect of obesity on spermatogenesis in those mice (Zhang et al., 2015). The incretin response to lipid has been reported to be dose-dependent (Yoder et al., 2009), which may explain why no effect was reported with the 10% Intralipid vehicle used in the Cammack et al. (2003) study using the same dosing schedule.

Parenteral administration of DEHP to experimental animals has led to mixed results regarding lung toxicity (FDA/CDRH, 2001), and one factor that could affect the results, in addition to age and dose, is the formulation administered. Greener et al. (1987) injected a single dose level of DEHP (168.4 mg/kg bw/day) solubilized in 4% bovine serum albumin (BSA) for 18 consecutive days starting on PND 3 and reported no treatment-related lung effects. Sjöberg et al. (1985) administered six infusions of a DEHP emulsion up to 500 mg/kg bw over a period of 12 days to 40-day old rats in an emulsion with a mean particle diameter less than 0.8 μm with “occasional particles” between 2 and 5 μm and found no lung lesions. In the present study, no granulomas were observed in vehicle controls, but lung granulomas were observed in the two highest iv dose groups at PND 7 and in all iv DEHP dose groups at PND 23. The particle size of the lipid emulsion was observed to increase with increasing DEHP dose (Supplemental Figure 4), and these larger lipid globules likely contributed to the presence of granulomas, although the magnitude of increase of large particles with increasing dose was not reflected in the incidence or severity of the granulomas in the PND 23 animals. Lipid injectable emulsions for clinical use are required to have less than 0.05% globules of a diameter greater than 5 microns for clinical use (Driscoll, 2009; Driscoll et al., 2006), as large particles can accumulate in capillaries of the lung and other organs. This particle size effect could have contributed also to the increase in spleen and liver weight to body weight ratios seen in the iv arm of the present study, as has been observed in other studies (Nordstrand et al., 1987). Microgranulomas in the lungs, livers, and spleens were also observed in adult rats infused for 28 days with emulsions of the

plasticizer DINCH in 20% Intralipid (David et al., 2015). Although lipid emulsion particle sizes were not reported in David et al. (2015), it is possible that incorporation of the test agent into the emulsion produced similar particle size effects to those reported here. Care would need to be taken when formulating DEHP or other plasticizers emulsions to ensure a particle size distribution similar to the 20% Intralipid vehicle control in order to clarify whether these agents contribute to granuloma formation or other lung lesions.

Several studies have reported a delay in lung development with decreased formation of alveolar septa in the offspring of dams treated orally with high doses of DEHP (> 1,000 mg/kg bw/day) during late gestation and lactation (Magliozzi et al., 2003; Rosicarelli and Stefanini, 2009) or a high dose (750 mg/kg bw/day) administered intraperitoneally to neonates (Liang et al., 2018). We observed a similar effect in the 600 mg DEHP/kg bw/day group of both exposure routes. This effect is like that observed in the bronchopulmonary dysplasia that is common in premature infants and that has complex causes, including exposure to high oxygen concentrations and mechanical respiration (Bancalari and Jain, 2019; Kalikkot Thekkeveedu et al., 2017). DEHP could potentially exacerbate this condition in the NICU, as has been suggested by others (Liang et al., 2018; Magliozzi et al., 2003; Rosicarelli and Stefanini, 2009), and while this effect was convincing only at the highest DEHP dose in this study, lower dose effects in the iv arm cannot be excluded. As with the induction of granulomas, this issue of lower dose effects could only be resolved if DEHP preparations with a similar particle size distribution to the Intralipid vehicle could be used and if morphometric assessments are conducted using lungs that are inflated at a consistent pressure at necropsy.

In summary, the results obtained in this study were largely consistent with those reported by Cammack et al. (2003) for the testicular toxicity of iv and oral DEHP administered to neonatal rats, although Sertoli cell toxicity not reported in that study was observed in this study and a clear NOEL was not seen at the lowest dose, 60 mg/kg bw/day, in the present study. Repeated oral administration of the lipid vehicle over this developmental period resulted in testicular toxicity; the relevance of this observation remains to be determined. The study confirms that a high oral dose of DEHP can delay the development of alveolar septa in the lung and this also occurs with iv administration, although interpretation of the lung effects after iv administration is confounded by increased particle size in the DEHP-containing lipid emulsions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Medical procedures using parenteral routes result in the highest human exposures to DEHP
- This study extended a previous study that compared the effects of oral and iv DEHP in developing rats
- Adverse effects in testis and possibly lung were observed in all DEHP dose groups and dosing routes
- Oral, but not iv, Intralipid vehicle produced adverse effects in testis suggesting gut mediation

A) 20% Intralipid, iv control

B) 20% Intralipid, oral control

Figure 1:

Representative photomicrographs of testis sections from PND 23 20% Intralipid intravenous (Panel A) and oral (Panel B) vehicle control rats. The oral controls had tubules containing numerous apoptotic spermatocytes and depletion of germinal epithelium (indicated by asterisks) that were not present in the iv controls. Original magnification, 200x.

A. Oral 20% Intralipid control

B. Oral 600 mg DEHP/kg bw/day

Figure 2:

Representative photomicrographs illustrating the apparent decrease in air spaces and alveolar septation in the lungs of PND 23 rats treated with 600 mg DEHP/kg bw/day *versus* vehicle controls. Oral vehicle control (Panel A) and 600 mg DEHP/kg bw/day (Panel B). Original magnification, 5x.

Table 1.

PND 7 Body and organ weights^{a, b, c}

Endpoint	DEHP dose (mg/kg bw/day)							
	Intravenous				Oral			
	0	60	300	600	0	60	300	600
Body Weight (g)	13.77 ± 1.86	13.65 ± 1.81	14.25 ± 1.72	13.55 ± 2.39	14.53 ± 1.29	13.50 ± 2.56	13.46 ± 1.68	11.48 ± 1.65*
Liver								
Absolute	423 ± 81	435 ± 64	549 ± 80	610 ± 163*	434 ± 55	450 ± 143	481 ± 68	413 ± 64
% bw	3.06 ± 0.26	3.18 ± 0.10	3.85 ± 0.32**	4.46 ± 0.59***	2.98 ± 0.16	3.28 ± 0.44	3.57 ± 0.21*	3.61 ± 0.43*
Kidney								
Absolute	172 ± 27	176 ± 22	184 ± 25	191 ± 33	185 ± 23	178 ± 33	164 ± 29	150 ± 20
% bw	1.25 ± 0.12	1.29 ± 0.09	1.29 ± 0.07	1.41 ± 0.13*	1.27 ± 0.08	1.32 ± 0.06	1.21 ± 0.08	1.31 ± 0.14
Lung								
Absolute	324 ± 65	283 ± 35	327 ± 54	322 ± 77	354 ± 41	293 ± 78	334 ± 77	249 ± 42*
% bw	2.34 ± 0.22	2.08 ± 0.19	2.29 ± 0.25	2.38 ± 0.37	2.44 ± 0.28	2.15 ± 0.20	2.49 ± 0.55	2.19 ± 0.40
Testis								
Absolute	19.2 ± 3.1	20.2 ± 0.9	18.8 ± 1.6	16.5 ± 1.7	21.5 ± 0.8	18.1 ± 1.9**	16.5 ± 2.3***	13.8 ± 1.9***
% bw	0.14 ± 0.02	0.15 ± 0.01	0.13 ± 0.01	0.12 ± 0.01	0.15 ± 0.01	0.14 ± 0.02	0.12 ± 0.02*	0.12 ± 0.01**

^aData are presented as mean ± S.D., n=6 except for the oral 300 mg/kg bw/day group, where n = 5. Body weight is given in g, all organ weights are mg.

^bAsterisks indicate a significant difference from the control of the same exposure route.

* p< 0.05;

** p<0.01;

*** p<0.001

^cThe means at each DEHP dose level in the oral and iv exposure arms were compared by t-tests with a Holm correction for multiple comparisons; there were no significant differences.

Table 2.

PND 23 Body and organ weights^{a, b, c}

Endpoint	DEHP dose (mg/kg bw/day)							
	Intravenous				Oral			
	0	60	300	600	0	60	300	600
Body Weight (g)	54.88 ± 5.94	55.97 ± 4.74	51.95 ± 4.62	46.43 ± 4.60 [*]	58.17 ± 5.25	55.33 ± 3.48	51.27 ± 4.29	42.23 ± 5.57 ^{***}
Brain								
Absolute (g)	1.42 ± 0.06	1.43 ± 0.05	1.37 ± 0.06	1.36 ± 0.06	1.45 ± 0.04	1.46 ± 0.04	1.43 ± 0.05	1.31 ± 0.07 ^{***}
% body wt	2.60 ± 0.23	2.56 ± 0.20	2.66 ± 0.14	2.95 ± 0.22 [*]	2.51 ± 0.28	2.64 ± 0.17	2.80 ± 0.25	3.14 ± 0.28 ^{***}
Liver								
Absolute (g)	2.32 ± 0.05	2.46 ± 0.02	2.52 ± 0.04	2.85 ± 0.04	2.53 ± 0.04	2.53 ± 0.03	2.47 ± 0.03	2.38 ± 0.03
% body wt	4.20 ± 0.50	4.39 ± 0.26	4.84 ± 0.38 [*]	6.12 ± 0.51 ^{***}	4.33 ± 0.44	4.58 ± 0.32	4.82 ± 0.36	5.63 ± 0.28 ^{***}
% brain wt	163.2 ± 29.3	172.2 ± 15.7	182.9 ± 18.5	208.6 ± 25.4 ^{**}	175.0 ± 33.2	174.2 ± 19.1	173.4 ± 22.4	180.3 ± 17.2
Kidney								
Absolute (mg)	603 ± 61	602 ± 55	586 ± 91	520 ± 40	644 ± 46	599 ± 44	548 ± 61 [*]	465 ± 69 ^{***}
% body wt	1.10 ± 0.05	1.08 ± 0.07	1.12 ± 0.08	1.12 ± 0.07	1.11 ± 0.06	1.08 ± 0.05	1.07 ± 0.05	1.10 ± 0.05
% brain wt	42.6 ± 2.8	42.1 ± 2.8	42.5 ± 5.1	38.2 ± 2.0	44.5 ± 3.8	41.2 ± 2.9	38.4 ± 4.4 [*]	35.3 ± 6.6 ^{***}
Lung								
Absolute (mg)	511 ± 69	482 ± 65	500 ± 67	485 ± 71	577 ± 100	522 ± 22	486 ± 82	479 ± 181
% body wt	0.93 ± 0.05	0.86 ± 0.05	0.97 ± 0.13	1.05 ± 0.16	0.99 ± 0.13	0.95 ± 0.07	0.95 ± 0.13	1.15 ± 0.47
% brain wt	36.0 ± 4.4	33.8 ± 4.3	36.4 ± 4.7	35.6 ± 5.2	39.9 ± 7.5	35.9 ± 2.1	34.0 ± 5.6	36.5 ± 13.8
Spleen								
Absolute (mg)	226 ± 30	249 ± 13	255 ± 41	240 ± 24	259 ± 45	225 ± 20	237 ± 40	170 ± 33 ^{***} ##
% body wt	0.41 ± 0.05	0.45 ± 0.05	0.49 ± 0.10	0.52 ± 0.03 [*]	0.45 ± 0.07	0.41 ± 0.05	0.46 ± 0.06	0.40 ± 0.03###
% brain wt	16.0 ± 1.8	17.5 ± 1.4	18.6 ± 3.2	17.6 ± 1.1	18.0 ± 3.4	15.4 ± 1.1	16.6 ± 2.9	12.8 ± 2.0 ^{**} ##
Testis								
Absolute (mg)	316 ± 39	287 ± 35	182 ± 25 ^{***}	154 ± 21 ^{***}	293 ± 38	248 ± 29 [*]	159 ± 15 ^{***}	120 ± 18 ^{***}

Endpoint	DEHP dose (mg/kg bw/day)							
	Intravenous				Oral			
	0	60	300	600	0	60	300	600
% body wt	0.58 ± 0.04	0.51 ± 0.06 [*]	0.35 ± 0.03 ^{***}	0.33 ± 0.02 ^{***}	0.50 ± 0.06	0.45 ± 0.05	0.31 ± 0.01 ^{***}	0.29 ± 0.03 ^{***} #
% brain wt	22.3 ± 2.7	20.1 ± 2.6	13.2 ± 1.6 ^{***}	11.3 ± 1.4 ^{***}	20.3 ± 2.8	17.0 ± 1.7 [*]	11.1 ± 1.0 ^{***}	9.1 ± 1.1 ^{***}
Epididymis								
Absolute (mg)	47.1 ± 10.7	46.7 ± 12.0	45.2 ± 9.3	41.7 ± 8.0	48.1 ± 6.8	44.0 ± 4.3	44.4 ± 7.2	36.3 ± 5.6 ^{**}
% body wt	0.09 ± 0.02	0.08 ± 0.02	0.09 ± 0.01	0.09 ± 0.02	0.08 ± 0.01	0.08 ± 0.00	0.09 ± 0.01	0.09 ± 0.01
% brain wt	3.3 ± 0.8	3.0 ± 0.3	3.1 ± 0.5	2.8 ± 0.3	3.3 ± 0.5	3.0 ± 0.3	3.1 ± 0.5	2.8 ± 0.3
Seminal vesicle								
Absolute (mg)	12.1 ± 2.8	14.2 ± 6.2	9.7 ± 2.4	7.3 ± 1.9	14.4 ± 3.0	11.0 ± 2.9 [*]	9.0 ± 0.9 ^{**}	9.6 ± 1.6 ^{**}
% body wt	0.02 ± 0.00	0.03 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.01
% brain wt	0.9 ± 0.2	1.0 ± 0.4	0.7 ± 0.2	0.5 ± 0.1	1.0 ± 0.2	0.8 ± 0.2 [*]	0.6 ± 0.1 ^{**}	0.7 ± 0.1 [*]
Prostate								
Absolute (mg)	53.5 ± 7.0	46.9 ± 8.2	53.9 ± 14.1	38.2 ± 5.8 [*]	43.3 ± 6.2	49.6 ± 5.7	37.0 ± 7.4	32.0 ± 7.5 [*]
% body wt	0.10 ± 0.01	0.08 ± 0.01	0.10 ± 0.02	0.08 ± 0.00	0.07 ± 0.01 [#]	0.09 ± 0.01	0.07 ± 0.02	0.08 ± 0.01
% brain wt	3.8 ± 0.5	3.3 ± 0.6	3.9 ± 0.9	2.8 ± 0.4 [*]	3.0 ± 0.4	3.4 ± 0.4	2.6 ± 0.5	2.4 ± 0.5

^aData are presented as mean ± S.D., n=6

^bAsterisks indicate a significant difference from the control of the same exposure route.

^{*}, p< 0.05;

^{**}, p<0.01;

^{***}, p<0.001

^cThe means at each DEHP dose level in the oral and iv exposure arms were compared by t-tests with a Holm correction for multiple comparisons. Significant differences are indicated in the oral columns with “#”.

[#], p< 0.05;

^{##}, p<0.01

Testis histopathology

Table 3.

Age	Diagnosis ^a	DEHP dose (mg/kg bw/day) ^b									
		Intravenous					Oral				
		0	60	300	600		0	60	300	600	
PND 7	Apoptosis, Sertoli cell	0/6	0/6	0/6	5/6*** 5/0/0 (1.0/0.8)		3/6 3/0/0 (1.0/0.5)	4/6 4/0/0 (1.0/0.7)	5/5## 4/1/0 (1.2/1.2)	5/6** 2/0/2/1 (2.4/2.0)	
	Apoptosis, gonocyte	2/6 1/1/0/0 (1.5/0.5)	2/6 2/0/0/0 (1.0/0.3)	3/6 1/2/0/0 (1.7/0.8)	3/6 1/2/0/0 (1.7/0.8)		5/6 5/0/0/0 (1.0/0.8)	4/6 4/0/0/0 (1.0/0.7)	5/5 5/0/0/0 (1.0/1.0)	4/6 3/1/0/0 (1.3/0.8)	
	Mitotic alteration, gonocyte	1/6 1/0/0/0 (1.0/0.2)	0/6	4/6 3/1/0/0 (1.3/0.8)	5/6** 3/1/1/0 (1.6/1.3)		0/6	2/6* 2/0/0/0 (1.0/0.3)	4/5** 1/2/1/0 (2.0/1.6)	5/6*** 1/1/2/1 (2.6/2.2)	
	Polynucleation, gonocyte	2/6 2/0/0/0 (1.0/0.3)	1/6 1/0/0/0 (1.0/0.2)	5/6* 0/1/3/1 (3.0/2.5)	6/6** 3/0/1/2 (2.3/2.3)		4/6 3/0/1/0 (1.5/1.0)	2/6 0/1/1/0 (2.5/0.8)	5/5* 0/0/2/3 (3.6/3.6)	5/6** 0/0/0/5 (4.0/3.3)	
	Hyperplasia, interstitial cell ^c	3/6 3/0/0/0 (1.0/0.5)	4/6 4/0/0/0 (1.0/0.7)	4/6 2/2/0/0 (1.5/1.0)	5/6* 2/3/0/0 (1.6/1.3)		3/6 3/0/0/0 (1.0/0.5)	3/6 2/1/0/0 (1.3/0.7)	5/5 4/1/0/0 (1.2/1.2)	4/6 3/1/0/0 (1.3/0.8)	
PND 23	Apoptosis, germ cell	4/6 4/0/0/0 (1.0/0.7)	4/6 4/0/0/0 (1.0/0.7)	3/6 3/0/0/0 (1.0/0.5)	6/6 5/1/0/0 (1.2/1.2)		5/6# 0/0/0/5 (4.0/3.3)	6/6# 1/1/0/4 (2.8/2.8)	6/6* 4/2/0/0 (1.3/1.3)	6/6** 5/1/0/0 (1.2/1.2)	
	Depletion, germinal epithelium	0/6	0/6	6/6*** 0/2/2/2 (3.0/3.0)	6/6*** 0/0/2/4 (3.7/3.7)		6/6## 2/4/0/0 (1.7/1.7)	6/6## 2/4/0/0 (1.7/1.7)	6/6** 0/0/6/0 (3.0/3.0)	6/6*** 0/0/0/6 (4.0/4.0)	
	Vacuolization cytoplasmic, multifocal, Sertoli cell	0/6	0/6	6/6*** 1/2/0/3 (2.8/2.8)	6/6*** 0/2/2/2 (3.0/3.0)		5/6# 0/2/3/0 (2.6/2.2)	4/6 1/1/2/0 (2.3/1.5)	6/6 0/3/3/0 (2.5/2.5)	6/6** 0/0/1/5 (3.8/3.8)	

^a Examples of the lesions diagnosed are shown in Supplemental Figures 1 (PND 7) and 2 (PND 23).^b Number of affected animals/total number of animals; number of animals with minimal/mild/moderate/marked lesion severity score; in parentheses, mean severity score in affected animals/mean severity score in all animals. Asterisks indicate statistically significant difference from the vehicle control in the same route of exposure arm, pound signs indicate statistically significant difference between the oral and iv exposure arms at a given dose level:

*, # p<0.05;

***, ## p<0.01;

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p<0.001

The diagnosis of hyperplasia required that the cluster of Leydig cells be at least half the size of a "median" size estimate for the smaller population of tubules. Those clusters that were at least half the size but not as large in diameter as the smaller tubules were given a score of 1; those aggregates that were the size of the smaller tubules or larger were given a score of 2. Any specimen that had at least one Leydig cell aggregate warranting a grade 2 got an overall score of 2.

Table 4.

PND 23 testis morphometry

Age	Endpoint	DEHP dose (mg/kg bw/day) ^a							
		Intravenous				Oral			
		0	60	300	600	0	60	300	600
PND 23	Seminiferous tubule diameter (µm)	150.7 ± 4.4	142.7 ± 13.4	125.7 ± 6.0 ^{***}	124.9 ± 4.2 ^{***}	143.1 ± 3.3 ^{b#}	135.2 ± 3.6 ^{b*}	122.6 ± 6.0 ^{b**}	112.4 ± 3.4 ^{b***##}
	Testicular tissue area ^c (mm ²)	15.7 ± 2.6	14.7 ± 1.7	11.1 ± 1.4 ^{***}	9.5 ± 1.2 ^{***}	14.6 ± 2.2	14.3 ± 2.1 ^b	10.2 ± 1.1 ^{b***}	8.6 ± 10 ^{***}

^aData shown are mean ± S.D. Asterisks indicate statistically significant difference from the vehicle control in the same route of exposure arm, pound signs indicate statistically significant difference between the oral and iv exposure arms at a given DEHP dose level:

^{*}, # p<0.05;
^{**}, ## p<0.01;
^{***} p<0.001.

^b n = 5 due to tissue artifact in specimens that prevented accurate measurements. All others n = 6.

^cTesticular tissue, mostly seminiferous tubular epithelium.

Table 5.

Lung histopathology

Age	Diagnosis	DEHP dose (mg/kg bw/day) ^a							
		Intravenous				Oral			
		0	60	300	600	0	60	300	600
PND 7	Eosinophilic crystals, multifocal	0/6	0/6	0/6	4/6** 4/0/0/0 (1.0/0.7)	0/6	0/6	0/5	0/6
	Granuloma, multifocal, alveolus	0/6	0/6	3/6* 2/1/0/0 (1.3/0.7)	5/6*** 2/2/1/0 (1.8/1.5)	0/6	0/6	0/5	0/6
	Hemorrhage, multifocal	0/6	0/6	0/6	4/6** 2/1/1/0 (1.8/1.2)	0/6	0/6	0/5	0/6
	Inflammation, interstitium	0/6	0/6	0/6	1/6 0/0/0/1 (4.0/0.7)	0/6	0/6	0/5	0/6
PND 23	Granuloma, multifocal, alveolus	0/6	5/6* 5/0/0/0 (1.0/0.8)	6/6*** 6/0/0/0 (1.0/1.0)	6/6*** 6/0/0/0 (1.0/1.0)	0/6	0/6	0/6	0/6
	Hemorrhage, multifocal	0/6	0/6	0/6	1/6 0/1/0/0 (2.0/0.3)	0/6	0/6	0/6	0/6
	Infiltration cellular, histiocyte, alveolus	0/6	0/6	1/6 0/1/0/0 (2.0/0.3)	1/6 1/0/0/0 (1.0/0.2)	0/6	0/6	0/6	2/6 1/1/0/0 (1.5/0.5)
	Inflammation, interstitium	0/6	0/6	0/6	2/6 0/1/0/1 (3.0/1.0)	1/6 1/0/0/0 (1.0/0.2)	0/6	0/6	0/6
	Developmental malformation, parenchyma	0/6	2/6 1/1/0/0 (1.5/0.5)	2/6 1/1/0/0 (1.5/0.5)	5/6** 3/2/0/0 (1.4/1.2)	1/6 1/0/0/0 (1.0/0.2)	1/6 1/0/0/0 (1.0/0.2)	2/6 0/2/0/0 (2.0/0.7)	4/6* 2/2/0/0 (1.5/1.0)

^a Number of affected animals/total number of animals; number of animals with minimal/mild/moderate/marked lesion severity score; in parentheses, mean severity score in affected animals/mean severity score in all animals. Asterisks indicate statistically significant difference from the vehicle control in the same route of exposure arm, pound signs indicate statistically significant difference between oral and iv exposure arms at a given DEHP dose level:

* p<0.05;

** ## p<0.01;

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