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Validation of an automated counting procedure for phthalate-induced testicular multinucleated germ cells

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Abstract

In utero exposure to certain phthalate esters results in testicular toxicity, characterized at the tissue level by induction of multinucleated germ cells (MNGs) in rat, mouse, and human fetal testis. Phthalate exposures also result in a decrease in testicular testosterone in rats. The anti-androgenic effects of phthalates have been more thoroughly quantified than testicular pathology due to the significant time requirement associated with manual counting of MNGs on histological sections. An automated counting method was developed in ImageJ to quantify MNGs in digital images of hematoxylin-stained rat fetal testis tissue sections. Timed pregnant Sprague Dawley rats were exposed by daily oral gavage from gestation day 17 to 21 with one of eight phthalate test compounds or corn oil vehicle. Both the manual counting method and the automated image analysis method identified di-*n*-butyl phthalate, butyl benzyl phthalate, dipentyl phthalate, and di-(2-ethylhexyl) phthalate as positive for induction of MNGs. Dimethyl phthalate, diethyl phthalate, the brominated phthalate di-(2-ethylhexyl) tetrabromophthalate, and dioctyl terephthalate were negative. The correlation between automated and manual scoring metrics was high ($r = 0.923$). Results of MNG analysis were consistent with these compounds' anti-androgenic activities, which were confirmed in an *ex vivo* testosterone production assay. In conclusion, we have developed a reliable image analysis method that can be used to facilitate dose-response studies for the reproducible induction of MNGs by *in utero* phthalate exposure.

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Conflict of Interest Statement

Kim Boekelheide does occasional expert consulting with chemical and pharmaceutical companies and owns stock in a small start-up biotechnology company (Semma Therapeutics) developing a cell-based therapy for diabetes.

Disclaimer

The research described in this article has been reviewed by the U.S. Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Keywords

phthalate esters; quantitative pathology; multinucleated germ cells

1. Introduction

Exposure to certain phthalic acid esters (phthalates) *in utero* disrupts male reproductive tract development. In the rat, these phthalates reproducibly reduce testosterone and induce a suite of characteristic testis histopathological alterations, including the induction of multinucleated germ cells (MNGs) in late gestation (Barlow and Foster, 2003; Boekelheide et al., 2009; Kleymenova et al., 2005; Mylchreest et al., 2002; Parks et al., 2000). However, the lack of a known molecular initiating event(s) has limited the understanding of phthalate toxicity in the male reproductive system (Howdeshell et al., 2015). Species differences in the magnitude of the anti-androgenic response to phthalates make it unlikely that the testicular histopathological alterations and testosterone reduction are mutually dependent outcomes. In fetal mouse testes and human fetal testis xenotransplant models, phthalates induce MNGs even in the absence of a statistically significant reduction in testicular testosterone or markers of testosterone biosynthesis (Gaido et al., 2007; Heger et al., 2012; Mitchell et al., 2012; Spade et al., 2014).

The anti-androgenic effects of phthalates are well-characterized in the rat *in utero* exposure model. In this model, phthalates with side chain lengths of C4 to C8 produce dose-dependent reductions in testicular testosterone (Furr et al., 2014), through a mechanism involving decreased expression of mRNAs involved in testosterone biosynthesis, including *Cyp11a1*, *Cyp17a1*, *Hsd3b1*, *Scarb1*, and *Star* (Hannas et al., 2012; Howdeshell et al., 2015; Johnson et al., 2007). Testicular testosterone can be measured readily in a large number of samples, and as a result the dose-response relationship between phthalate exposures and these anti-androgenic effects have been thoroughly characterized. However, the MNG induction resulting from phthalate exposure has not previously been quantified on a large scale because of the low throughput nature of manually counting cells on digital slides. The number of MNGs has not been evaluated for many phthalates, and there is little dose-response data on the number of MNGs even for those phthalates which have been tested.

The current study had two primary goals. First, we sought to develop a counting method with sufficient throughput to enable the quantification of phthalate-induced MNGs in many samples. Second, we aimed to assess whether induction of MNGs by phthalates would correspond with known anti-androgenic effects on a per-compound basis. To achieve these goals, we developed an automated image analysis method in ImageJ to count MNGs in images of fetal testis histological slides. We then applied the automated image analysis method to samples exposed to vehicle or high doses of eight phthalate compounds known to be positive or negative for anti-androgenic effects.

2. Materials and Methods

2.1. Animals

This study was conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee of the National Health and Environmental Effects Research Laboratory (USEPA). Timed pregnant Sprague Dawley rats (CrI:CD(SD); strain code: 001) were obtained from Charles River (Raleigh, NC). Dams were mated on postnatal day 90, and the day on which a vaginal plug was detected was considered gestational day (GD) 0. Rats were housed individually in clear polycarbonate cages (20 × 25 × 47 cm) with heat-treated, laboratory grade pine shavings and fed NIH07 rodent diet and filtered (5 µm) municipal tap water *ad libitum*. Dams were weight-ranked and randomly assigned to treatment groups to produce similar mean weights. Animals were housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and maintained at 20–22°C, 45–55% humidity, and a 12:12 h photoperiod (lights off at 18:00).

2.2. Chemicals and Exposure Protocol

Dimethyl phthalate (DMP), diethyl phthalate (DEP), di-*n*-butyl phthalate (DBP), butyl benzyl phthalate (BBP), dipentyl phthalate (DPeP), and di-(2-ethylhexyl) phthalate (DEHP) were gifted to USEPA from the National Toxicology Program (Research Triangle Park, NC) and independently verified at 100% purity by Research Triangle Institute (Durham, NC) using gas chromatography with flame ionization detection. Di-(2-ethylhexyl) tetrabromophthalate (TBPH) was purchased from Unitex Chemical Corporation (Greensboro, NC) and dioctyl terephthalate (DOTP) was purchased from Aldrich (Milwaukee, WI). Timed pregnant rats were exposed to one of the eight treatment compounds at the dose given in Table 1, or corn oil (Sigma-Aldrich; St. Louis, MO) vehicle in a 2.5 mL/kg body weight dosing solution by daily oral gavage from GD 17–21. Maternal weight was recorded daily during the dosing period. Three pregnant dams were exposed to each compound except DMP (2 dams) and vehicle (6 dams). Doses were selected to exceed the concentration at which each compound is known to induce MNGs or a reduction in testicular testosterone production. DBP and DEHP are known to induce MNGs at doses of at least 100 mg/kg/d and 135 mg/kg/d, respectively (Andrade et al., 2006; Boekelheide et al., 2009; Johnson et al., 2008; Kleymenova et al., 2005; Mahood et al., 2007). DBP, BBP, DPeP, and DEHP reduce testicular testosterone with ED50 between 35 and 200 mg/kg/d in Harlan Sprague Dawley rats (Furr et al., 2014). DMP, DEP, TBPH, and DOTP do not significantly reduce testosterone and were chosen as negative controls to allow comparisons between anti-androgen positive and negative compounds for the MNG endpoint. Dams were euthanized by decapitation on GD 21, 1 h after the final dose was administered. During necropsy, the total number of live and dead fetuses and number of resorptions was recorded for each litter. Testes were isolated from male fetuses using a dissecting microscope. For histological analysis, one testis from each of up to four males per litter was collected and held in Hank's Balanced Salt Solution (HBSS) temporarily until fixation. Testes were fixed in modified Davidson's fixative for 15 m, then transferred to 70% ethanol and stored at 4°C. One testis from each of three males per litter was isolated for an *ex vivo* testosterone production assay.

2.3. Histology

Fixed testes were processed through a series of graded ethanols and embedded in paraffin. An average of 3.58 testes per litter were embedded, with all testes from the same litter in a single block. Paraffin blocks were trimmed to the approximate center of the sample, and 5 μm sections were cut and mounted on glass slides. Sections were then deparaffinized in xylene and rehydrated through a series of graded ethanols. One section from each block was stained with hematoxylin and eosin (H&E) for manual scoring, and one was stained with hematoxylin alone for automated image analysis. Digital slide images were obtained by scanning slides on an Aperio ScanScope CS (Leica Biosystems, Buffalo Grove, IL) at 40 $\times$ objective magnification.

2.4. Image Analysis

For manual scoring of scanned slides, digital images of H&E-stained slides were annotated by a scorer, who was blinded to the treatment, using Aperio ImageScope software. 104 images were scored, with an average of 3.58 images (testes) per litter. Because MNG rates have been reported in the literature using several different metrics, manual scores were quantified using four different approaches to determine whether statistical significance was reproducible across metrics. Images were scored for the total cross-sectional testis area, seminiferous cord cross-sectional area, the total number of MNGs, the number of seminiferous cord cross sections visible in the whole testis section, and the number of MNGs in each seminiferous cord cross section. Annotation data were exported as text files and summarized using Excel as (1) the number of MNGs per unit testis cross-sectional area (MNGs/ mm^2 testis), (2) number of MNGs per total seminiferous cord cross-sectional area (MNGs/ mm^2 cord), (3) average number of MNGs per seminiferous cord cross section (MNGs/cord), and (4) percent of seminiferous cord cross sections with at least one MNG (% cords with MNGs). The percent of total testis cross-sectional area occupied by seminiferous cords was also determined.

Automated image analysis was performed on digital images of scanned hematoxylin-stained slides (whole testis cross-sections) (Fig. 1). Hematoxylin stain, without eosin, was chosen for this procedure because hematoxylin-stained slide images can be converted to binary images with more consistency than H&E slides. Snapshots of each testis were captured in ImageScope software at screen resolution at 10.0–12.4 $\times$ digital magnification. 100 total images were analyzed, with an average of 3.45 images (testes) per litter. Digital images were imported into ImageJ and processed as follows. Images were subjected to batch-specific RGB thresholding and converted to 16-bit binary. The “Fill Holes” command was applied to increase pixel density of nuclei. The “Despeckle” command was applied to remove stray particles close to nuclei that could affect the final particle analysis. Small and irregular particles were removed using batch-specific cutoffs for size (ranging from 0 to 500 pixels) and circularity (ranging from 0 to 0.15). MNGs were identified as objects with size greater than 165 μm^2 and circularity ranging from 0.17 to 1. These size and circularity thresholds can be adjusted based on the characteristics of the image set. Total testis area was also measured on the binary-converted image, relative to the scale bar embedded in the image. Image analysis was performed on the entire dataset of 100 whole testis cross-section images in two separate batches of 49 and 51 images. Sensitivity and false discovery rate were

determined by hand-checking automated MNG identifications on 20 randomly selected images. Data were summarized using Excel, as MNGs/mm² testis cross-sectional area.

2.5. Ex vivo testosterone production assay

One testis was isolated from each of three separate male fetuses in each litter and used to measure testosterone production (three technical replicates per litter). Testes isolated on GD 21 were incubated at 37°C for 3 hr in 500 µL of M-199 media in 24-well plates under gentle agitation. After incubation, the media were collected and stored at -80°C until further analyses. Testosterone concentrations were measured in incubation media using radioimmunoassay (Testosterone RIA, ALPCO, Salem, NH) according to manufacturer specifications.

2.6. Statistical analysis

For all analyses, values obtained from males in the same litter were averaged, and the litter was treated as the statistical unit. The sample size was 3 litters for all treatment groups except DMP (2 litters). For MNG, maternal weight, and litter size data, vehicle control was treated as a single group with a sample size of 6 litters. For testosterone RIA data, samples were statistically compared to concurrent controls only, with a sample size of 3 litters. MNG data was analyzed in Prism v7 (GraphPad, La Jolla, CA) by one-way ANOVA, followed by Holm-Sidak *post hoc* test. Pearson correlation analysis was also performed in Prism. Analysis of testosterone production, maternal weight, and litter size data was performed in SAS (v. 9.4, SAS Institute, Cary, NC), using the GLM procedure, followed by pairwise comparison using LSMEANS test. $p < 0.05$ was considered significant in all analyses. Charts were drawn in Prism, and data are represented as mean $\pm$ SEM.

3. Results

3.1. BBP, DBP, DEHP, and DPeP induce MNGs in fetal rat testes

Following phthalate exposure from GD 17–21, no significant differences in litter size were observed between any phthalate treatment group and control, and no fetal losses or resorptions were noted in any group. Maternal weight at termination of the study was not reduced in any group, but maternal weight gain was reduced by an average of 35.8 g in DPeP exposure (Supplemental Table 1). MNGs were clearly visible in H&E-stained sections of testes in the DBP, BBP, DPeP, and DEHP exposure groups. Samples treated with vehicle control, DMP, DEP, TBPH, and DOTP were largely devoid of MNGs (Fig. 2). A total of 765 MNGs were identified using the manual counting approach. The induction of MNGs in DBP, BBP, DPeP, and DEHP-treated testes was statistically significant (Fig. 3), regardless of whether the MNG rate was quantified as the number of MNGs per unit testis area, MNGs per unit seminiferous cord area, percent of cord cross sections with MNGs, or average number of MNGs per cord cross section (Fig. 3A–D). The background rate of MNGs in vehicle control samples and negative control phthalate samples (DMP, DEP, TBPH, and DOTP) was 1.91, 2.35, 3.35, 3.88, and 0.43 MNGs/mm² testis, respectively, compared to 60.01, 64.13, 108.86, and 75.50 MNGs/mm² testis for DBP, BBP, DPeP, and DEHP, respectively. There were no significant differences in MNG rate between vehicle controls and negative control phthalates. There were no significant differences in the proportional

seminiferous cord area in testis sections (Fig. 3E), and all metrics for MNG rate were strongly correlated (Fig. 3F–H).

3.2. Automated scoring of MNGs

The automated scoring procedure identified the same four phthalate treatment groups, BBP, DBP, DEHP, and DPeP, as significantly inducing MNGs relative to vehicle-treated control (Fig. 4A). Manually counted MNGs correlated strongly with the counts derived from the automated image analysis (Pearson $r = 0.943$) (Fig. 4B). A total of 570 MNGs were identified by automated analysis of 100 images. Sensitivity and false discovery rate were determined by manually confirming MNG identifications on 20 randomly selected images. The sensitivity of the image analysis algorithm, defined as (automatically identified MNGs/automatically identified MNGs + false negatives) was 73.5%. The false discovery rate, defined as (false positives/automatically identified MNGs + false positives) was 2.4%. For each batch of images processed, initial threshold adjustments can be performed in 10 to 30 minutes, after which the entire batch can be processed in less than a minute. Occasional errors occur due to variations in staining intensity. These errors can be corrected for by individually adjusting thresholds for the samples in question.

3.3. Testosterone production

The four phthalate treatment groups that significantly induced MNGs (BBP, DBP, DPeP, and DEHP), also significantly decreased *ex vivo* testosterone production, by 69.2%, 75.4%, 74.9%, and 62.4%, respectively, relative to vehicle control (Fig. 5). DOTP exposure also unexpectedly resulted in a statistically significant 30.5% reduction in *ex vivo* testosterone production.

4. Discussion

Eight compounds were tested in this study for induction of MNGs in the fetal rat testis. Exposure by oral gavage from GD 17–21 induced MNGs in the DBP, BBP, DPeP, and DEHP treatments, while DMP, DEP, TBPH, and DOTP treatments resulted in no significant induction of MNGs (Figs. 2–3). This effect was detected using an automated image analysis algorithm in ImageJ, which gave a high correlation between manual and automated counting methods (Fig. 4). The sensitivity of the automated counting procedure was estimated at 73.5%. The algorithm failed to detect some MNGs because they were immediately adjacent, resulting in two MNGs being counted as one. However, others may have been undetected because the nuclei were not in contact or for other reasons the algorithm was unable to detect the association between the nuclei. More importantly, the algorithm was highly specific. The rate of false discoveries was only 2.4%, indicating that the counting procedure excluded the majority of true negatives, including both mononuclear germ cells and other nuclei. While there were some false negative errors that decreased the total MNG count, they did not prevent the automated analysis from detecting induction of MNGs with statistical significance. Notably, the ImageJ procedure can be adjusted for batch-to-batch variation in staining intensity by adjusting color thresholding, size, and circularity parameters. To further validate the accuracy of the image analysis method, we applied several different rate calculations that have been reported throughout the phthalate literature to the manual MNG

counts, including the number of MNGs per cross-sectional area of testes or cords, the number of MNGs per cord cross section, and the proportion of cord cross sections with at least one MNG. These measures were all strongly correlated (Fig. 3), indicating that the number of MNGs per unit testis cross-sectional area would be as accurate as any other metric. This is important for validation of the counting procedure, as cross-sectional testis area is by far the most practical denominator for automated quantification.

The induction of MNGs by DBP and DEHP was consistent with previously published data. However, to our knowledge, this is the first report evaluating MNG induction following BBP or DPeP exposure. DBP and DEHP are well-known to induce MNGs. In multiple studies, DBP induces MNGs in the fetal rat testis at doses of 100 mg/kg/d or greater, but MNGs have not been observed at doses at or below 50 mg/kg/d (Boekelheide et al., 2009; Johnson et al., 2008; Kleymenova et al., 2005; Mahood et al., 2007). DEHP doses of at least 135 mg/kg/d have induced MNGs in the rat, but doses at or below 45 mg/kg/d have not (Andrade et al., 2006). While most studies involve daily exposure by oral gavage throughout multiple days of gestation, high-dose DBP exposure induced MNGs in as little as 24 h in studies where exposure began on or after GD 18 (Ferrara et al., 2006; Spade et al., 2015). Induction of MNGs by BBP could also be predicted based on its metabolism. The active metabolite responsible for phthalate toxicity is the monoester produced by cleavage of a single side chain, predominantly by intestinal lipase (Ozaki et al., 2017). The active metabolites of DBP and DEHP are MBP and MEHP, respectively. For BBP, the predominant metabolite excreted in rat urine is MBP, though mono-*n*-benzyl phthalate (MBeP) is also formed (Nativelle et al., 1999). The metabolism of BBP to MBP is therefore consistent with induction of MNGs by BBP.

Each of the eight compounds tested in this study has been previously tested for reduction of testosterone production in fetal rat testes. Ortho phthalates with aliphatic side chain length between 4 and 8 carbons reduce testosterone, with the greatest effect being observed in compounds with 4 to 6 carbon chains, including DBP, BBP, DPeP, and DEHP. DMP, DEP, TBPH, and DOTP do not reduce testosterone (Furr et al., 2014). Correspondingly, exposure to DBP, BBP, DPeP, and DEHP, but not DEP, DMP, or DOTP inhibits masculinization of male rat fetuses, which encompasses reduced fetal or neonatal anogenital distance (AGD) at or below the doses used in the present study (Barlow and Foster, 2003; Gray et al., 2016; Gray et al., 2000; Mylchreest et al., 1998). AGD was not measured in the present study, as the relationship between fetal testosterone reduction and AGD has been previously characterized for these compounds. Both BBP metabolites, MBP and MBeP, are known to produce anti-androgenic effects in fetal rats (Ema et al., 2003; Shono et al., 2000). The effects of phthalates on testosterone production in the present study largely confirmed previously reported findings, with DBP, BBP, DPeP, and DEHP significantly reducing testosterone production (Fig. 5). Therefore, within the subset of phthalates tested in the present study, induction of MNGs corresponded with the known anti-androgenic activity of each compound. Further, the dose-response curves for the two sets of effects are likely similar, based on the lowest observed effect doses for MNGs in published studies of DBP (100 mg/kg/d) and DEHP (135 mg/kg/d), which compare closely to ED₅₀ values between 150–200 mg/kg/d for reduction of testosterone production in Harlan Sprague Dawley rats (Furr et al., 2014).

All of the negative results for testosterone production in this study corresponded with previously published results, with the exception that DOTP exposure resulted in a statistically significant reduction in testosterone production (Fig. 5). However, this positive result for DOTP appears to be spurious and may have resulted from the inherent variability of the RIA data. The magnitude of reduction in testosterone production was only 30.5%, relative to control, or approximately half of the reduction caused by the known positive phthalates. In a previous experiment, DOTP exposure resulted in no significant reduction in testosterone and no effects on masculinization of male fetuses (Gray et al., 2000). Of the negative compounds, DMP and DEP have side chain lengths of one and two carbons, respectively, placing them outside the range of side chain lengths associated with anti-androgenic activity. DOTP and DEHP both share the di-(2-ethylhexyl) side chain with DEHP. However, DOTP is a para-phthalate, and the phthalic acid group in TBPH is brominated. Notably, tetrabromo MEHP (TBMEHP), the monoester metabolite of TBPH, has induced MNGs significantly at doses that did not significantly reduce testosterone (Springer et al., 2012). However, conversion of TBPH to the monoester TBMEHP is slow relative to metabolism of DEHP to MEHP. Therefore, both the histopathological and anti-androgenic response to TBPH could be limited by the low *in vivo* metabolic rate. A future study will consider the *in vivo* dose-response to TBMEHP for MNG and anti-androgenic endpoints.

This study demonstrates that there is concordance between the anti-androgenic activity of a phthalate and its induction of MNGs, a hallmark of phthalate-induced histopathological alterations in the fetal testis. Dose-responses for MNG induction remain to be performed. However, we can conclude that for each tested compound, in utero exposure to a high dose leads to both a decrease in testosterone and induction of MNGs, or is negative for both effects. It remains unclear how these two effects are linked. Most likely, phthalates have a single molecular target that separately induces different downstream effects based on actions in different cell types. The ability of phthalates to induce MNGs in mice and humans without a significant reduction of testicular testosterone (Heger et al., 2012; Johnson et al., 2012; Mitchell et al., 2012), as well as the lack of MNG induction following abiraterone exposure in human fetal testis (Spade et al., 2014), indicate that MNG induction and testosterone reduction are unlikely to be mutually dependent. We further conclude that the image analysis algorithm created in ImageJ succeeded in accurately automating MNG counting. Manual counts and automated counts from the same samples gave the same result with strong correlation (Fig. 4). Despite the fact that *in utero* phthalate exposure reproducibly results in a suite of testicular histopathological alterations including induction of MNGs, dose-response data on testicular pathology are limited, and the majority of published studies on this outcome focus on only DBP and DEHP. This is largely due to the low throughput of manually counting histopathological phenomena such as MNGs. The automated MNG counting method is significantly less labor- and time-intensive than manual MNG counting, and will enable quantification of MNG induction in dose response studies of multiple phthalates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Developed an ImageJ procedure to count multinucleated germ cells (MNGs).
- Four medium-chain ortho-phthalates induced MNGs in fetal rat testis.
- For each compound, induction of MNGs was concordant with testosterone reduction.

Figure 1. Automated MNG counting in ImageJ

Original images of hematoxylin-stained rat fetal testis were processed in ImageJ by converting to binary images, applying the “Fill Holes” and “Despeckle” commands, and filtering out objects below size and circularity thresholds. MNGs are identified by size and circularity in the processed images. Identified MNGs are highlighted in yellow in the final panel. Inset area defined by dashed line in original image. Scale bar = 200 μ m.

Figure 2. Representative images of testicular histology following phthalate exposure

Fetal testis sections stained with hematoxylin and eosin. MNGs (arrowheads) were induced by DBP, BBP, DPeP, and DEHP treatments (*), but not DMP, DEP, TBPH, or DOTP. Scale bar = 60 μ m.

Figure 3. Significant induction of MNGs by DBP, BBP, DPeP, and DEHP

DBP, BBP, DPeP, and DEHP significantly induced MNGs, relative to vehicle control. This induction was significant whether MNGs were quantified as the number of MNGs per unit total testis area (MNGs/mm² testis) (A), number of MNGs per unit seminiferous cord area (MNGs/mm² cord) (B), percent of cords with MNGs (C), or the average number of MNGs per cord (MNGs/cord) (D). The ratio of cord to testis area was not significantly different between groups (E). As a result, the MNGs/mm² testis and MNGs/mm² seminiferous cord values were highly correlated (Pearson $r = 0.994$) (F). MNGs/mm² testis was also highly correlated with the percent of cords with MNGs ($r = 0.975$) (G) and the number of MNGs per cord ($r = 0.973$) (H). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by one-way ANOVA followed by Holm-Sidak multiple comparison test. Data in A-E are presented as mean $\pm$ SEM.

Figure 4. Significant induction of MNGs identified by automated image analysis

Image analysis in ImageJ found the rate of MNGs per unit area of testis section to be significantly greater than control in DBP, BBP, DPeP, and DEHP treatments (A). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ by one-way ANOVA followed by Holm-Sidak multiple comparison test. Data are represented as mean $\pm$ SEM. Automated image analysis data (auto) were strongly correlated with manual counts of MNGs from H&E stained slides (manual), with Pearson $r = 0.923$ (B). Dashed line = identity.

Figure 5. Effects of in utero phthalate exposure on fetal testis testosterone production

Ex vivo testis testosterone production was significantly reduced by DBP, BBP, DPeP, DEHP, and DOTP treatments. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ by LSMEANS test. Data are represented as mean $\pm$ SEM.

Table 1

Exposure compounds.

Common Name	Abbreviation	Chain Length [*]	CAS Number	Dose (mg/kg/d)
dimethyl phthalate	DMP	1	131-11-3	900
diethyl phthalate	DEP	2	84-66-2	900
di- <i>n</i> -butyl phthalate	DBP	4	84-74-2	750
butyl benzyl phthalate	BBP	4	85-68-7	750
dipentyl phthalate	DPeP	5	131-18-0	300
di-(2-ethylhexyl) phthalate	DEHP	6	117-81-7	750
di-(2-ethylhexyl) tetrabromophthalate	TBPH	6	26040-51-7	750
dioctyl terephthalate	DOTP	6	6422-86-2	750

* number of carbons in parent aliphatic side chain