OXFORD
SOT | Society of Toxicology
www.toxsci.oxfordjournals.org

TOXICOLOGICAL SCIENCES, 152(1), 2016, 244–256

doi: 10.1093/toxsci/kfw079
Advance Access Publication Date: April 27, 2016
Research Article

Multiple Endocrine Disrupting Effects in Rats Perinatally Exposed to Butylparaben

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ABSTRACT

Parabens comprise a group of preservatives commonly added to cosmetics, lotions, and other consumer products. Butylparaben has estrogenic and antiandrogenic properties and is known to reduce sperm counts in rats following perinatal exposure. Whether butylparaben exposure can affect other endocrine sensitive endpoints, however, remains largely unknown. In this study, time-mated Wistar rats (n = 18) were orally exposed to 0, 10, 100, or 500 mg/kg bw/d of butylparaben from gestation day 7 to pup day 22. Several endocrine-sensitive endpoints were adversely affected. In the 2 highest dose groups, the anogenital distance of newborn male and female offspring was significantly reduced, and in prepubertal females, ovary weights were reduced and mammary gland outgrowth was increased. In male offspring, sperm count was significantly reduced at all doses from 10 mg/kg bw/d. Testicular *CYP19a1* (aromatase) expression was reduced in prepubertal, but not adult animals exposed to butylparaben. In adult testes, *Nr5a1* expression was reduced at all doses, indicating persistent disruption of steroidogenesis. Prostate histology was altered at prepuberty and adult prostate weights were reduced in the high dose group. Thus, butylparaben exerted endocrine disrupting effects on both male and female offspring. The observed adverse developmental effect on sperm count at the lowest dose is highly relevant to risk assessment, as this is the lowest observed adverse effect level in a study on perinatal exposure to butylparaben.

Key words: reproduction; paraben; testis; breast; prostate; sexual development; endocrine disruption.

Parabens are preservatives commonly used in cosmetics and lotions, and some also in foods. Dermally applied parabens are absorbed by the skin and metabolized by esterases, conjugated and excreted in urine and bile (reviewed by Soni *et al.*, 2005). Although metabolism following dermal and oral exposure is rapid, toxic effects have been reported in experimental studies on oral exposure to some parabens, including butylparaben.

Some parabens have estrogenic properties both *in vitro* and *in vivo*, and butylparaben has been shown to affect endocrine-sensitive organ systems (Boberg *et al.*, 2010). Studies on juvenile rats exposed to butylparaben have shown conflicting results, as reduced sperm count and testosterone level reported in one study at doses from 10 mg/kg bw/d (Oishi, 2001) was not corroborated by subsequent studies investigating similar dose levels (Gazin *et al.*, 2013; Hoberman *et al.*, 2008).

Stronger evidence is found regarding effects on sperm count after developmental exposure to parabens (Kang *et al.*, 2002; Yang *et al.*, 2015; Zhang *et al.*, 2014). Reduced epididymal sperm count and motility were seen in rat offspring of dams exposed subcutaneously to 100 and 200 mg butylparaben/kg bw/d during gestation and lactation (Kang *et al.*, 2002). In a recent study, perinatal exposure of rats to 2.5 mg/kg bw/d of isobutylparaben reduced epididymal sperm count and motility compared with control (Yang *et al.*, 2015). In another rat study, oral exposure to *n*-butylparaben at doses of 0, 64, 160, 400, and 1000 mg/kg/d from gestation day (GD) 7 to postnatal day (PND) 21 led to reduced male anogenital distance (AGD) at PND 1 and 21, delayed preputial separation (PPS), reduced reproductive organ weights at several ages, and reduced epididymal sperm count at PND 90 at the 2 highest dose levels (Zhang *et al.*, 2014). These effects were associated with reduced testosterone and LH levels, and elevated estradiol and progesterone levels in serum from prepubertal male rats, supporting the view that butylparaben can exert both antiandrogenic and estrogenic effects on the developing organism. The underlying mechanisms for the effect on sperm count remain unknown, but in addition to estrogenic properties (Byford *et al.*, 2002; Routledge *et al.*, 1998), *in vitro* studies have shown inhibition of androgen receptor activation by butylparaben (Chen *et al.*, 2007; Satoh *et al.*, 2005).

Little is known about effects of butylparaben on other endocrine sensitive endpoints such as prostate and mammary gland development. Estrogenic chemicals have been shown to affect early mammary gland development in female rodents and to accelerate mammary gland growth. For instance, an increase in outgrowth and number of terminal end buds (TEBs) in prepubertal female rat mammary glands have been shown after exposure to estrogenic compounds such as ethinyl estradiol and genistein (Cotroneo *et al.*, 2002; Mandrup *et al.*, 2012, 2015). These findings may predict adverse effects on human breast development and possibly increased risk of breast cancer following early exposure to estrogenic chemicals (Soto *et al.*, 2013). Animal studies have shown that developmental exposure to endocrine disrupting compounds, or elevated perinatal levels of estrogens, can induce atypical hyperplasia of the prostatic epithelium, as well as morphological changes resembling those of precancerous lesions in humans (prostatic intra-epithelial neoplasia, PIN lesions) (Prins *et al.*, 2007).

Due to the endocrine disrupting activity of butylparaben, a possible influence on prostate and mammary development can be hypothesized, and hence this study aimed to investigate developmental effects of butylparaben on these organs in prepubertal and adult offspring. We also intended to improve risk assessment of butylparaben by investigating endocrine sensitive endpoints in perinatally exposed rats at doses from 10 mg/kg bw/d, and to elucidate the mechanisms underpinning the influence of butylparaben on sperm count.

MATERIALS AND METHODS

Test compounds

Butylparaben (purity >99.0%, CAS no. 94-26-8) was purchased from Sigma-Aldrich (Brøndby, Denmark). Corn oil was used both as a control compound and vehicle and purchased from Sigma (Brøndby, Denmark).

Animals and exposure

Seventy-two time-mated nulliparous, young adult Wistar rats (HanTac:WH, SPF, Taconic Europe, Ejby, Denmark) were supplied at day 3 of pregnancy. The day when a vaginal plug was detectable was designated as GD 1 and the expected day of delivery, GD 23 was designated as pup day (PD) 1. The study was performed in 2 blocks of 36 dams (separated by 1 week), and all dose groups were equally represented in both blocks. The animal experiment was carried out at the DTU National Food Institute (Mørkhøj, Denmark). Ethical approval was obtained from the Danish Animal Experiments Inspectorate: authorization number, 2012-15-2934-00089 C4. The experiments were overseen by the National Food Institutes in-house Animal Welfare Committee for animal care and use.

The dams were housed in pairs until GD 17 and alone thereafter under standard conditions in semi-transparent polysulfone (PSU) cages (PSU 80-1291HOOSU Type III, Tecniplast) (15 × 27 × 43 cm) with aspen wood chip bedding (Tapvei, Denmark), Enviro Dri nesting material (Brogaarden, Lynge, Denmark) and aspen wood shelters Tapvei Arcade 17 (Brogaarden, Lynge, Denmark). The PSU bottles and cages, as well as the aspen wood shelters (instead of polycarbonate) were used to reduce the risk of migration of bisphenol A that potentially could confound the study results. The animal room had controlled environmental conditions with a 12-h light/dark cycle with light intensity 500 lux starting at 9 PM, humidity 55% ± 5, temperature at 21 ± 1 °C and ventilation changing air 10 times per hour. All animals were fed on a standard diet with ALTROMIN 1314 (soy- and alfalfa-free, ALTROMIN GmbH, Lage, Germany). Acidified tap water (to prevent microbial growth) in PSU bottles (84-ACBTO702SU Tecniplast) were provided ad libitum. After weaning, offspring were housed in pairs.

The day after arrival (GD 4), time-mated dams were distributed into 4 groups of 18 rats with similar body weight (bw) distributions. The dams received vehicle (controls), or 10, 100, or 500 mg/kg bw/d of butylparaben. Test compounds and vehicle were administered by oral gavage with a stainless steel probe 1.2 × 80 mm (Scanbur, Karlslunde, Denmark) once daily in the morning from GD 7 to the day before expected birth (GD 21) and again after birth from PD 1 to 22 at a constant volume of 2 ml/kg bw/d. Dams that did not give birth were omitted from the study. The exposure period was chosen to cover the most sensitive windows of reproductive development in rat offspring. The individual doses were based on the bw of the animal on the day of dosing. The dams were inspected twice a day for general toxicity including changes in clinical appearance. Body weights were recorded on GD 4 and daily during the dosing period to monitor changes in weight gain, to follow pregnancy status and to adjust dose according to weight.

In vivo measurements

The day after delivery the pups were counted, sexed, weighed, and checked for anomalies. Dead pups were investigated macroscopically for pathological changes when possible. AGD was measured in all offspring using a stereomicroscope with unit markings on the ocular. All offspring were weighed on PD 6. On PD 14, all male and female pups were weighed and examined for number of areolas/nipples (NR), described as a dark focal area (with or without a nipple bud) located where nipples are normally present in female offspring. The same skilled technician, blinded with respect to exposure groups, recorded both AGD measurements and NR counts. After weaning of the offspring at PD 22, the dams were killed and the number of implantation sites was registered to calculate postimplantation and perinatal loss.

Pubertal onset was assessed by determining day of vaginal opening (VO) or the day of balano-PPS in weaned female and male offspring, respectively. Registrations were performed daily in females from PD 27 until VO was detected in all animals. Males were examined daily from PD 39 until the last male was positive. Age and bw of the rats were recorded on the day in which VO and balano-PPS was first observed. Onset of puberty was assessed blinded with respect to exposure groups by skilled technicians.

Necropsy of male and female offspring PD 16/17 and 22

Reproductive organ weights and histological and gene expression changes in reproductive organ weights were examined at PD 16 (males) and 17 (females), as this age has proved sensitive for detection of particularly anti-androgenic effects of chemicals (Christiansen *et al.*, 2009; Metzdorff *et al.*, 2007). On PD 16 and 17, one male and one female pup from each litter (n = 12–18) were weighed and decapitated, and blood was collected in heparinized tubes for hormone analysis. From males the following organs were excised and weighed: Testes, ventral prostate, epididymis, seminal vesicle, levator ani/bulbocavernosus muscle (LABC), bulbourethral gland, liver, adrenal, and retroperitoneal fat pad. From females the following organs were excised and weighed: Ovary, liver, thyroid, and retroperitoneal fat pad. One testis per pup was placed in RNAlater for gene expression studies as described below).

On PD 22, one female and one male pup per litter (n = 12–14) were weighed and decapitated, and blood was collected in heparinized tubes for hormone analysis. From females, ovaries were excised and weighed, and from males testes and ventral prostate were excised and weighed. Testes were fixed in Bouin's fixative overnight and ventral prostates were fixed in formalin for histological examination. Mammary glands were dissected from female pups for whole mounting. The tissue was dissected in order to include the abdominal (4th) mammary gland with the adjacent lymph nodes and part of the inguinal (5th) mammary gland. At PD 22 the number of TEBs in rat mammary glands is peaking, and this age is considered sensitive to detection of changes in early mammary development (Mandrup et al., 2012; Russo et al., 1979).

Necropsy of adults and epididymal sperm count

Approximately one female and one male pup per litter were sacrificed at PD 80–90 and bws were determined. From males the following organs were excised and weighed: Testes, epididymis, seminal vesicle with prostate, ventral prostate (separated from dorsolateral prostate and seminal vesicle) LABC, bulbourethral gland, liver, thyroid, 4th mammary gland, and retroperitoneal fat pad. One testis per animal was fixed in Bouin's fixative overnight, and the contralateral was frozen for gene expression studies. Epididymides, ventral prostates, and mammary glands were fixed in formalin for histological examination.

For sperm count analysis, samples were analyzed using computer-assisted sperm analysis (CASA) system (HTM-IVOS, Hamilton Thorne Research, Massachusetts). From male offspring, alternately left or right cauda epididymis including 1 cm of ductus deferens was frozen in liquid nitrogen and stored at −80 °C for sperm count analysis. The cauda epididymis was thawed, weighed and prepared as described by Jarfelt et al. (2005), and samples were analyzed using a 10 × UV fluorescent objective and IDENT OPTIONS on the CASA. Ten fields were analyzed for each sample and 3 counts were performed for each suspension. Counts were averaged and data are presented as number of sperm per gram cauda. Sperm cell motility and morphology was not investigated.

Females were sacrificed on the day of estrous as judged from a vaginal smear in the morning. The following organs were excised and weighed: Ovaries, uterus, liver, thyroid, mammary glands (4th gland), and retroperitoneal fat pad. Abdominal (fourth) mammary glands, ovaries, and uteri were fixed in formalin for histological examination.

Hormone analysis and gene expression

Serum estradiol was measured in 8–10 plasma samples per dose group in PD 16 males and PD 22 females using Enzyme Linked Assay (Cayman kit no. 582251, Bertinpharma, Montigny-le-Bretonneux, France).

Gene expression analyses were performed on testes PD 16 (n = 9–10) and PD 90 (n = 4) by RT-qPCR. Protocols and verification of suitable reference genes for data normalization were as previously described (Svingen et al., 2015). Briefly, total RNA was extracted from the entire testis at PD16 or approximately 120 mg tissue (median cross-section) at PD90 using an RNA Mini kit (Qiagen), including on-column DNAse I treatment. RNA was subsequently quantified and purity-verified on a nano-drop spectrophotometer and 500 ng total RNA used to synthesize cDNA (Omiscript kit; Qiagen) in the presence of 6 μM Random Primer mix (New England Biolabs). cDNA was diluted 1:20 and used at 3 μl per RT-qPCR reactions. TaqMan Gene Expression Assays (Life Technologies) were: *Ddx4* (Rn01489814_m1), *Fshr* (Rn01648507_m1), *Hsd3b1* (Rn01774741_m1), *Lhr* (Rn00564309_m1), *Nr5a1* (Rn00584298_m1), *Sox9* (Rn01751069_mH), *Cyp19a1* (Taxvig et al., 2008), and *Ar*, *Cyp11a1*, *Cyp17a1* (Laier et al., 2006). RT-qPCR assays were run on a 7900HT Fast Real-Time PCR System (Applied Biosystems) over 45 cycles using a 2-step cycling protocol with annealing temp at 60 °C. Relative transcript abundance was calculated by the comparative Ct-method using the reference genes *Rpl13a* (Rn00821946_g1) and *Sdha* (Rn00590475_m1). Intra-assay variability of technical replicates was < 0.5 cycles.

Mammary whole mounts

Mammary glands from PD 22 female offspring were placed on a glass slide and stained with alum carmine as described in Mandrup et al. (2015). Briefly, the whole mounts were scanned on a flatbed scanner (4800 dpi) and mammary development was assessed on the digital images. Measurements were performed in Image Pro Plus 7.0 software (Media Cybernetics, Bethesda, Maryland). The glands were evaluated for outgrowth and extent of mammary development. For each dose group, 12–14 samples were evaluated. Outgrowth was assessed by measuring the outer area, longitudinal growth, transverse growth, distance to the lymph node (shortest distance from the 4th gland to the adjacent lymph node) and distance to the 5th gland (shortest distance from the 4th to the 5th abdominal mammary gland). The number of TEBs was counted (defined as tear-drop shaped buds in zone C with a diameter of 100 μm or more, as defined by Russo and Russo (1996a).

Histological examination

Female mammary glands PD 80–90 were sectioned and stained with haematoxylin and eosin by standard procedures. Histologic evaluation included ductular changes, lobular changes and functional changes in females confirmed to be in oestrous. Ductular changes included the evaluation and distribution of intraductal hyperplasia (defined as duct epithelium with 3 or more layers of epithelial cells as described by Singh et al., 2000). Evaluation of lobular changes included lobule types (as single alveoli/lobules smaller than type 1, lobules type 1 or lobules type 2 as defined by Russo et al., 1996a for humans) and lobuloalveolar structure of the lobules (loss of the typical tubuloalveolar architecture of alveoli). Finally, the distribution of secretory material in the ducts was evaluated as a sign of secretory activity.

Ovary histology was examined in one section from all adult females from the control and high-dose groups and focused only on health status and cyclic activity. No quantitative measures were made. Uterine histology was examined only to confirm estrous cycle stage to aid the histological examination of mammary glands.

Histological examination of one section per organ (testes, epididymides, and ventral prostate) was performed in all adult males from the high dose and control groups. A detailed qualitative examination of the testes was performed taking into account the tubular stages of the spermatogenic cycle to identify effects such as missing germ cell layers or types, retained spermatids, multinucleate or apoptotic germ cells and sloughing of spermatogenic cells into the lumen. Epididymides were evaluated for ductal atrophy, changes in sperm content and presence of sloughed testicular germ cells and cell debris in the epididymal lumen. Ventral prostates of adult males were evaluated with regard to degree of inflammation, epithelial atrophy, and atypical hyperplasia. Additionally, morphometric examination was made in ventral prostate from adult and prepubertal (PD 22) males to identify possible changes in the relative areas of different compartments. Each area was applied a grid with 15 points that were manually assigned as epithelium, stroma, lumen, or outside tissue section/undefined. This was done in 10 randomly selected areas per tissue section under 20 or 10× magnification (PD 22 and 90, respectively) using Image-Pro1 Plus ver. 7.0 (Media Cybernetics, Inc., USA). The percentage of areas occupied by each compartment (epithelium, stroma, and lumen) was compared between exposed groups and controls, and the ratio between epithelium and lumen was calculated and compared between groups. Grid points outside the tissue or undefined were not included in the statistical analysis.

Statistics

GraphPad Prism 5 was used for analysis of gene expression data and morphometrical data, whereas SAS Enterprise Guide 4.3 was used for all other data.

Data from continuous endpoints were examined for normal distribution and homogeneity of variance, and if relevant, log transformed. Data with normal distribution and homogeneity of variance were analyzed using analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Organ weights were analyzed using bw as a covariate. When more than one pup from each litter was examined, statistical analyses were adjusted using litter as an independent, random and nested factor in ANOVA or litter means were used. In cases where normal distribution and homogeneity of variance could not be obtained by data transformation (ie, whole mount measurements), a nonparametric Kruskall-Wallis test was applied followed by Dunn's multiple comparison test comparing exposed groups with controls.

AGD and organ weights were analyzed using bw as a covariate. The number of nipple/areolas was assumed to follow a binomial distribution with a response range between 0 and θ_{max}, with θ_{max} being equal to the biologically possible maximal number of nipples in rats, either 12 or 13. The choice of θ_{max} was decided on considering the global fit (information criterion of Schwarz). To account for litter effects on nipple retention, correlation structures between number of nipple/areolas and litter were modelled by the Generalized Estimating Equations method using the SAS procedure PROC GENMOD.

Histological scoring data were evaluated using the 2 × 2 Fisher's Exact Test without correction for multiple comparisons.

RESULTS

Dams and Delivery

Exposure to butylparaben did not affect maternal bw gain, gestation length, litter size, pre-, or perinatal offspring survival, or bws either at birth or in the postnatal period (Table 1).

In Vivo Markers of Endocrine Disrupting Effects

Endocrine-disrupting effects of butylparaben were seen in neonatal pups, as AGD (analyzed with bw as a covariate) was reduced dose-dependently in both males and females at PD 1 following perinatal exposure to 100 or 500 mg/kg bw/d (Figure 1 and Table 1). No significant effects were seen for male nipple retention (Table 1).

Sexual maturation, measured as day of VO in females and day of PPS in males occurred slightly later in both male and female offspring exposed to butylparaben, but this was not statistically significant (Table 1).

Organ Weights, Epididymal Sperm Counts, and Histology

No effects of butylparaben on the bw of offspring were seen on PD 16, 17, 22, or in adulthood. In male offspring, no changes in reproductive organ weights were detected at PD 16 or 22 (Table 2). In female offspring, ovary weights were reduced dose-dependently at PD 17, and the effect was statistically significant at 100 and 500 mg/kg bw/d (Figure 1; Table 2). Ovary weights were slightly higher compared with controls at PD 22, but this was not statistically significant ($P = .086$ at high dose) (Table 2).

In adult males (PD 90), the weight of prostate and seminal vesicle (together) and the weight of ventral prostate (separated from seminal vesicle) were reduced in the highest dose group compared with controls ($P = .015$ and .05, respectively) (Figure 1; Table 2). Epididymal weight was significantly increased in the middle dose group only ($P = .01$) (Table 2), and epididymal sperm count was significantly decreased in all dosed groups compared with the controls ($P < .0001$) (Figure 2).The histological examination of testes and epididymides from adult controls and high-dose males showed no differences between groups.

No other effects were seen on adult male or female organ weights (Table 2).

Testicular Gene Expression

At PD 16, *Cyp19a1* expression was significantly lower in testes from all exposure groups compared with controls (Figure 3a). There was a tendency towards highest levels of exposure showing lowest level of expression; relative mRNA levels were 33%, 30% and 23% that of control group for 10, 100, and 500 mg/kg bw/d groups, respectively. Expression level of several cell-specific marker genes was unchanged at PD16 following exposure to butylparaben. The germ cell marker *Ddx4* was unchanged in all groups, suggesting that germ cell numbers were unchanged (Figure 3a). The Sertoli cell markers *Sox9* and *Fshr* showed stable expression across groups, indicating that, at least Sertoli cell numbers were unaffected by exposure (Figure 3a). So too, the Sertoli/Leydig cell marker *Nr5a1* and *Ar* were unchanged, as were all the Leydig cell markers *Lhr*, *Cyp11a1*, *Cyp17a1*, and *Hsd3b1* (Figure 3a).

In adult testes, *Cyp19a1* expression was similar in exposed and control animals. All other marker genes were also unchanged except *Nr5a1*, which was lower than controls in all exposed groups (Figure 3b).

Histological Examination of Prostate

In ventral prostates of prepubertal males, morphometric analysis showed that the epithelial area (Figure 4A) and the ratio between epithelium and lumen (Figure 4B) were decreased in the middle dose group compared with controls. At this age, most

TABLE 1. Pregnancy and Litter Data of Dams and Offspring Exposed to 0, 10, 100, or 500 mg Butylparaben/kg bw/d from GD 7 to PD 22

Dams and Litters	Control	10 mg BP	100 mg BP	500 mg BP
No. of viable litters	n = 16	n = 13	n = 15	n = 17
Dam BW gain GD 7–21 (g)	78.73 ±16.6	81.04 ± 17.0	86.13 ± 11.2	75.34 ± 19.2
Dam BW gain GD 7-PND 1 (g)	15.67 ± 7.6	13.46 ± 7.9	13.83 ± 10.7	9.50 ± 10.0
Dam BW gain PND 1–17 (g)	31.56 ± 12.3	39.08 ± 1.3	37.33 ± 11.5	36.65 ± 12.7
Gestation length (days)	22.94 ± 0.3	23.00 ± 0.0	23.0 ± 0.0	23.03 ± 0.3
% postimplantation loss	8.98 ± 11.2	6.83 ± 6.0	8.9 ± 11.7	5.92 ± 10.6
% perinatal loss	13.48 ± 17.3	7.37 ± 7.0	8.90 ± 11.7	7.39 ± 12.2
Litter size	10.00 ± 3.5	11.92 ± 1.9	10.6 ± 3.1	10.47 ± 2.9
% perinatal deaths	4.50 ± 16.1	0.64 ± 2.3	0.0 ± 0.0	1.67 ± 5.1
% males	47.45 ± 23.8	49.97 ± 11.5	56.45 ± 12.8	50.45 ± 17.8
Offspring				
Mean male birth weight (g)	6.41 ± 0.4	6.45 ± 0.4	6.62 ± 0.3	6.31 ± 0.6
AGD males (mm)	3.96 ± 0.1	3.98 ± 0.1	**3.77 ± 0.2****	**3.69 ± 0.3*****
AGDI males (mm/g^{-3})	2.13 ± 0.1	2.14 ± 0.1	**2.01 ± 0.1*****	**2.00 ± 0.1*****
Mean female birth weight (g)	6.13 ± 0.3	6.04 ± 0.4	6.29 ± 0.3	5.98 ± 0.6
AGD females (mm)	2.22 ± 0.1	2.16 ± 0.1	**2.07 ± 0.1****	**2.01 ± 0.2*****
AGDI females (mm/g^{-3})	1.22 ± 0.0	1.19 ± 0.1	**1.12 ± 0.1****	**1.11 ± 0.1*****
Nipples (areolas) males	0.27 ± 0.35	0.15 ± 0.28	0.33 ± 0.39	0.54 ± 0.72
Nipples (areolas) females	12.3 ± 0.78	12.3 ± 0.3	12.3 ± 0.28	12.3 ± 0.25
Mean bw PD 6 (g)	13.2 ± 1.6	12.4 ± 1.1	12.8 ± 1.4	12.2 ± 1.4
Mean bw PD14 (g)	28.6 ± 4.0	26.1 ± 2.5	26.1 ± 4.2	26.6 ± 3.6
Mean bw PD 24 (g)	48.3 ± 6.7	44.0 ± 4.5	46.2 ± 6.6	45.8 ± 6.1
Age at VO	31.3 ± 2.8	33.1 ± 3.1	32.5 ± 2.4	33.2 ± 2.9
Bw at VO	78.1 ± 13	83.7 ± 14	80.8 ± 15	84.3 ± 13
Age at PPS	43.6 ± 1.4	44.1 ± 1.7	44.3 ± 1.9	44.7 ± 2.0
Bw at PPS	144 ± 12	148 ± 11	153 ± 13	155 ± 16

Data represent group means based on litter means ± SD.

prostates had small acini with columnar epithelium and an abundant amount of secretory material in the apical part of the cells (Figure 4C), whereas animals with a low ratio between epithelium and lumen had an increased incidence of large acini with cuboidal epithelium (Figure 4D).

Adult ventral prostate histology differed markedly between individuals, but this was not dose related. Several animals showed variable degree of inflammation in the ventral prostate, consisting of focal or diffuse accumulation of mononuclear cells. We observed no difference between groups in total numbers of animals with interstitial inflammation (5 out of 16 controls and 7 out of 18 high-dose animals), nor between groups in scores for degree of inflammation (data not shown). In some cases, the epithelium adjacent to inflammatory foci showed minor reactive hyperplasia with cellular atypia (4 out of 16 controls and 6 out of 18 high-dose animals). There was no difference between groups in scores for epithelial height, and morphometric analysis of the ratio between luminal area and epithelial area showed no differences between dose groups at PD 90 (data not shown).

Dorsolateral prostate showed a much lower incidence of inflammatory foci than ventral prostate, with small inflammatory foci only seen in 2 controls and 1 high-dose animal. Dark granules in epithelial cell cytoplasm or nuclei were seen in several areas of dorsolateral prostate for some animals, but no difference in incidence was seen between dose groups (7 out of 16 controls and 5 out of 18 high-dose animals affected).

Mammary Gland Development and Histology in Female Offspring

Prepubertal mammary glands were affected by perinatal exposure to butylparaben. At PD 22, female mammary glands showed a significantly higher number of TEBs in the 2 highest dose groups compared with controls (Figs. 4F–H). Indications of increased outgrowth towards the lymph node was seen, as the distance between mammary tissue and lymph node was significantly reduced in females exposed to 100 mg/kg bw/d of butylparaben compared with control females (Figure 4E).

No clear effect of butylparaben was seen on mammary glands of adult female offspring. However, more females displayed delayed differentiation of the adult mammary glands and increased proliferation of duct epithelium in the lowest dose group compared with controls, but this was not statistically significant. Five out of 13 females from the low dose group had less developed lobules (lobules smaller than type 1) compared with 1 of 14 females from the control group ($P = .08$ in 2 × 2 Fisher). Three out of 13 females had intraductal hyperplasia (>30% of tissue) in the low dose group, whereas no controls had intraductal hyperplasia ($P = .1$ in 2 × 2 Fisher) (Figs. 4I and J). No sign of secretory activity or changes in tubuloalveolar morphology of the lobules were observed.

Histological Examination of Ovaries

The histological examination of ovaries from adult controls and high dose females showed apparently healthy ovaries with presence of all stages of follicles and with an abundant number of corpora lutea (data not shown).

Serum Hormone Levels

Serum estradiol levels were examined at PD 16 in males and at PD 22 in females, i.e. time points when we observed effects that may be related to altered estradiol production (reduced

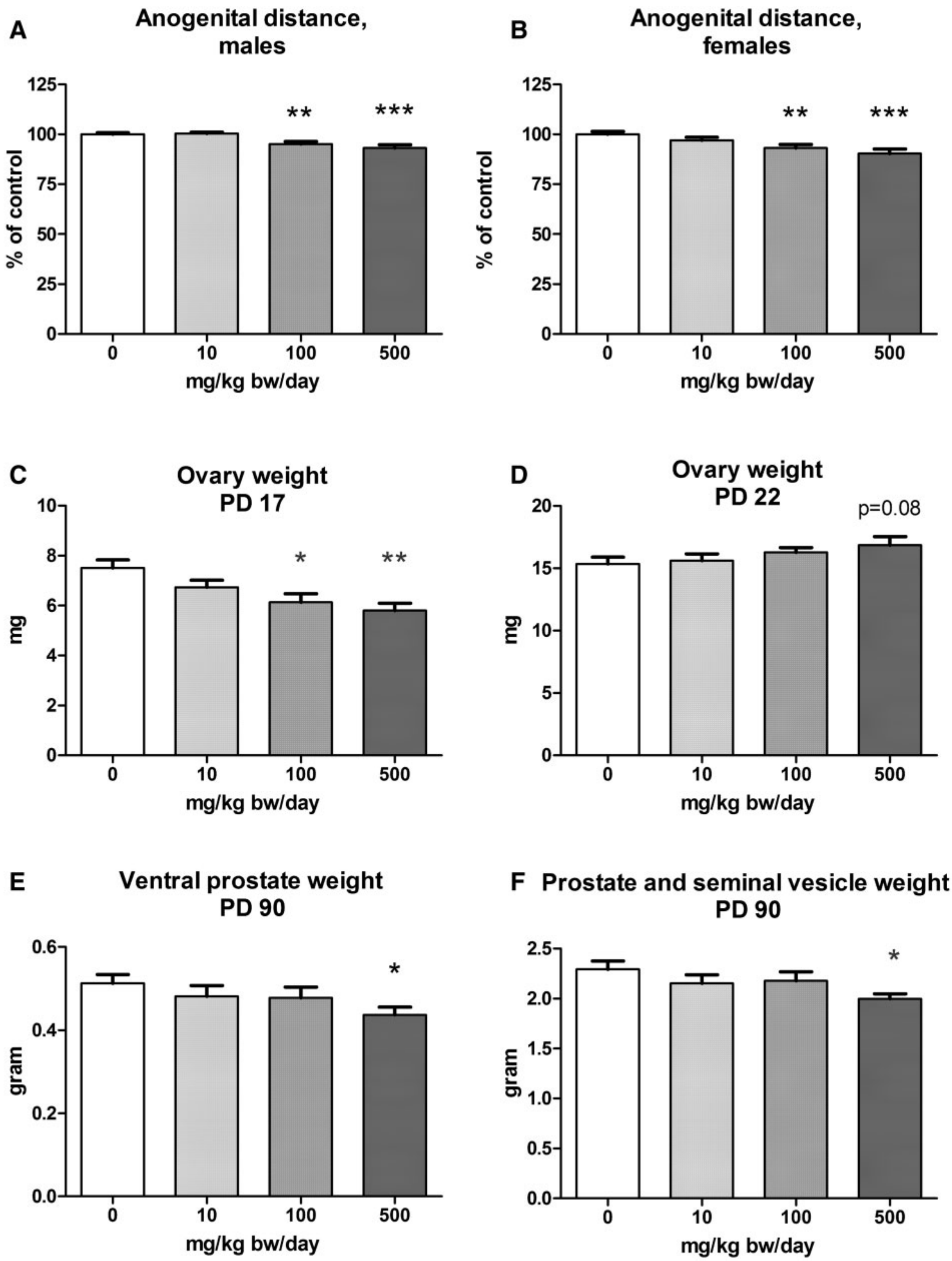

FIG. 1. Morphological effects in butylparaben-exposed rats. **A and B,** Perinatal exposure to 100 or 500 mg/kg bw/d of butylparaben caused shortened anogenital distance (AGD) in both male and female offspring relative to control animals. **C and D,** Ovary weights were reduced at PD 17, but not at PD 22, following perinatal exposure to 100 or 500 mg/kg bw/d of butylparaben. **E and F,** The weights of ventral prostate, total seminal vesicle and prostate were reduced at PND 90 following perinatal exposure to 500 mg/kg bw/d of butylparaben. Mean + SEM. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$. AGD was analyzed with bw as a covariate and litter as a random factor; organ weights were analyzed with bw as a covariate (n = 13–17 litters per dose group).

TABLE 2. Body and Organ Weights in Rat Offspring Following Perinatal Exposure to Butylparaben

	Control	Butylparaben 10	Butylparaben 100	Butylparaben 500
PD 16 males				
N	12	13	14	18
Body weight (g)	32 ± 5.0	30 ± 2.6	29 ± 4.4	30 ± 5.1
Liver (mg)	848 ± 167	796 ± 104	748 ± 142	805 ± 154
Retroperitoneal fat pad (mg)	49.7 ± 21.5	45.6 ± 10.9	42.5 ± 14.8	47.9 ± 18.4
Right testis (mg)	54.0 ± 9.3	56.4 ± 8.1	52.3 ± 8.6	54.0 ± 9.6
Left testis (mg)	55.6 ± 8.4	57.9 ± 7.9	52.9 ± 9.5	54.4 ± 9.5
Ventral prostate (mg)	12.5 ± 2.7	12.0 ± 2.4	12.2 ± 3.7	12.4 ± 2.8
Seminal vesicle (mg)	7.93 ± 2.79	8.60 ± 2.16	9.32 ± 2.96	8.36 ± 1.87
Epididymis (mg)	24.7 ± 3.9	24.8 ± 3.3	24.4 ± 4.2	25.2 ± 3.8
LABC (mg)	27.7 ± 3.6	30.1 ± 5.7	29.8 ± 7.9	32.5 ± 8.8
Bulbourethral gland (mg)	1.67 ± 0.37	2.02 ± 0.65	1.99 ± 0.48	1.95 ± 0.37
Adrenal (mg)	10.1 ± 2.9	9.5 ± 0.9	9.0 ± 2.2	9.8 ± 2.0
PD 17 females				
N	14	13	14	17
Body weight (g)	34 ± 4.6	30 ± 3.9	31 ± 4.2	32 ± 5.0
Liver (mg)	931 ± 143	819 ± 112	843 ± 128	867 ± 173
Retroperitoneal fat pad (mg)	38.1 ± 12.0	29.5 ± 13.1	31.7 ± 13.8	37.0 ± 14.8
Right ovary (mg)	3.58 ± 0.68	3.29 ± 0.63	2.91 ± 0.72	**2.72 ± 0.65****
Left ovary (mg)	3.93 ± 0.61	3.45 ± 0.54	**3.21 ± 0.66***	**3.08 ± 0.60*****
Pooled ovary weight (mg)	7.51 ± 1.19	6.74 ± 1.02	**6.13 ± 1.27***	**5.80 ± 1.21*****
Thyroid (mg)	5.14 ± 1.01	5.34 ± 1.42	5.71 ± 1.70	5.45 ± 1.94
PD 22 males				
N	11	12	13	14
Body weight (g)	45 ± 6.7	46 ± 4.6	46 ± 4.0	47 ± 6.0
Right testis (mg)	116 ± 14	125 ± 17	124 ± 11	124 ± 20
Left testis (mg)	116 ± 15	125 ± 16	125 ± 12	124 ± 19
Prostate (mg)	30.8 ± 3.9	31.5 ± 3.5	34.3 ± 5.7	34.5 ± 5.0
PD 22 females				
N	14	13	12	13
Body weight (g)	45 ± 5.5	44 ± 4.4	46 ± 4.9	43 ± 3.8
Right ovary (mg)	7.59 ± 0.95	7.90 ± 1.20	8.28 ± 0.81	8.48 ± 1.30
Left ovary (mg)	7.76 ± 1.37	7.70 ± 0.99	8.01 ± 0.86	8.37 ± 1.24
PD 80-90 males				
N	16	14	15	18
Body weight (g)	339 ± 26.8	327 ± 23.5	333 ± 27.8	336 ± 33.7
Liver (g)	10.9 ± 1.1	10.7 ± 1.1	10.8 ± 1.5	10.6 ± 1.2
Retroperitoneal fat pad (g)	2.09 ± 0.6	1.69 ± 0.60	1.87 ± 0.48	2.17 ± 0.93
Right testis (g)	1.75 ± 0.17	1.70 ± 0.11	1.81 ± 0.20	1.77 ± 0.15
Left testis (g)	1.79 ± 0.15	1.76 ± 0.15	1.86 ± 0.19	1.82 ± 0.15
Ventral prostate (g)	0.51 ± 0.08	0.47± 0.10	0.48 ± 0.10	**0.44 ± 0.08***
Seminal vesicle with prostate (g)	2.29 ± 0.31	2.12 ± 0.31	2.21 ± 0.32	**2.00 ± 0.20***
LABC (g)	0.965 ± 0.161	0.945 ± 0.097	0.939 ± 0.120	0.892 ± 0.082
Bulbourethral gland (g)	0.107 ± 0.020	0.113 ± 0.017	0.098 ± 0.024	0.097 ± 0.020
Adrenal (g)	0.508 ± 0.040	0.513 ± 0.043	0.547 ± 0.050	0.509 ± 0.045
PD 80-90 females				
N	15	14	16	18
Body weight (g)	218 ± 14.5	209 ± 14.8	215 ± 11.8	214 ± 15.9
Liver (g)	6.57 ± 0.68	6.31 ± 0.59	6.60 ± 0.65	6.75 ± 0.68
Retroperitoneal fat pad (g)	1.14 ± 0.27	0.92 ± 0.27	1.00 ± 0.25	1.00 ± 0.25
Right ovary(g)	0.048 ± 0.009	0.043 ± 0.004	0.045 ± 0.004	0.046 ± 0.006
Left ovary (g)	0.045 ± 0.007	0.042 ± 0.004	0.049 ± 0.008	0.045 ± 0.007
Uterus (g)	0.396 ± 0.061	0.382 ± 0.059	0.404 ± 0.046	0.394 ± 0.061
Thyroid (g)	0.023 ± 0.009	0.018 ± 0.004	0.018 ± 0.003	0.018 ± 0.007

LABC: levator ani/bulbocavernosus muscle. Asterisks indicate statistically significant difference from control group. *$P < .05$, **$P < .01$, ***$P < .001$.

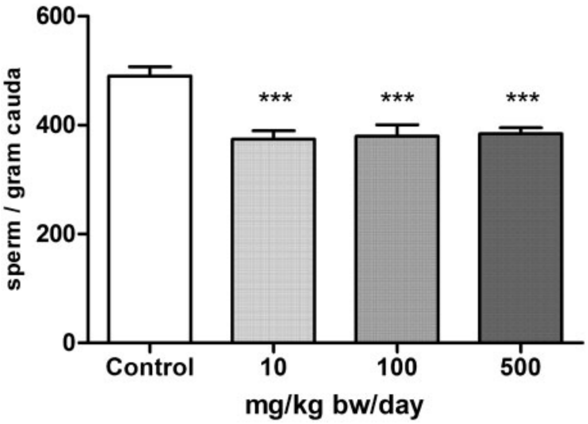

FIG. 2. Number of sperm per gram cauda in male rats exposed to 0, 10, 100, and 500 mg butylparaben/kg bw/d from GD 7 to PD 22. Group mean values ± SD are shown, n = 13–17. Sperm count is significantly lower, indicated by *** with $P < .0001$, in all dosed groups compared with the control group.

aromatase gene expression in testes and increased female mammary outgrowth). No effects of treatment were observed at these time points.

DISCUSSION

Butylparaben is known to affect sperm count in rat offspring, but until now knowledge on other reproductive endpoints has been lacking. In this study we found endocrine disrupting effects in both male and female offspring after perinatal exposure of rats, including shortened AGD, reduced sperm count and reproductive organ weight, disrupted gene expression in the testes, and abnormal mammary gland development. Of particular importance for risk assessment is the observation that the sperm count was lower at all doses, starting at 10 mg/kg.

Mechanisms of Actions for Effects on the Male Reproductive System

We observed a reduction in epididymal sperm count, which corroborates previous findings in rat offspring following

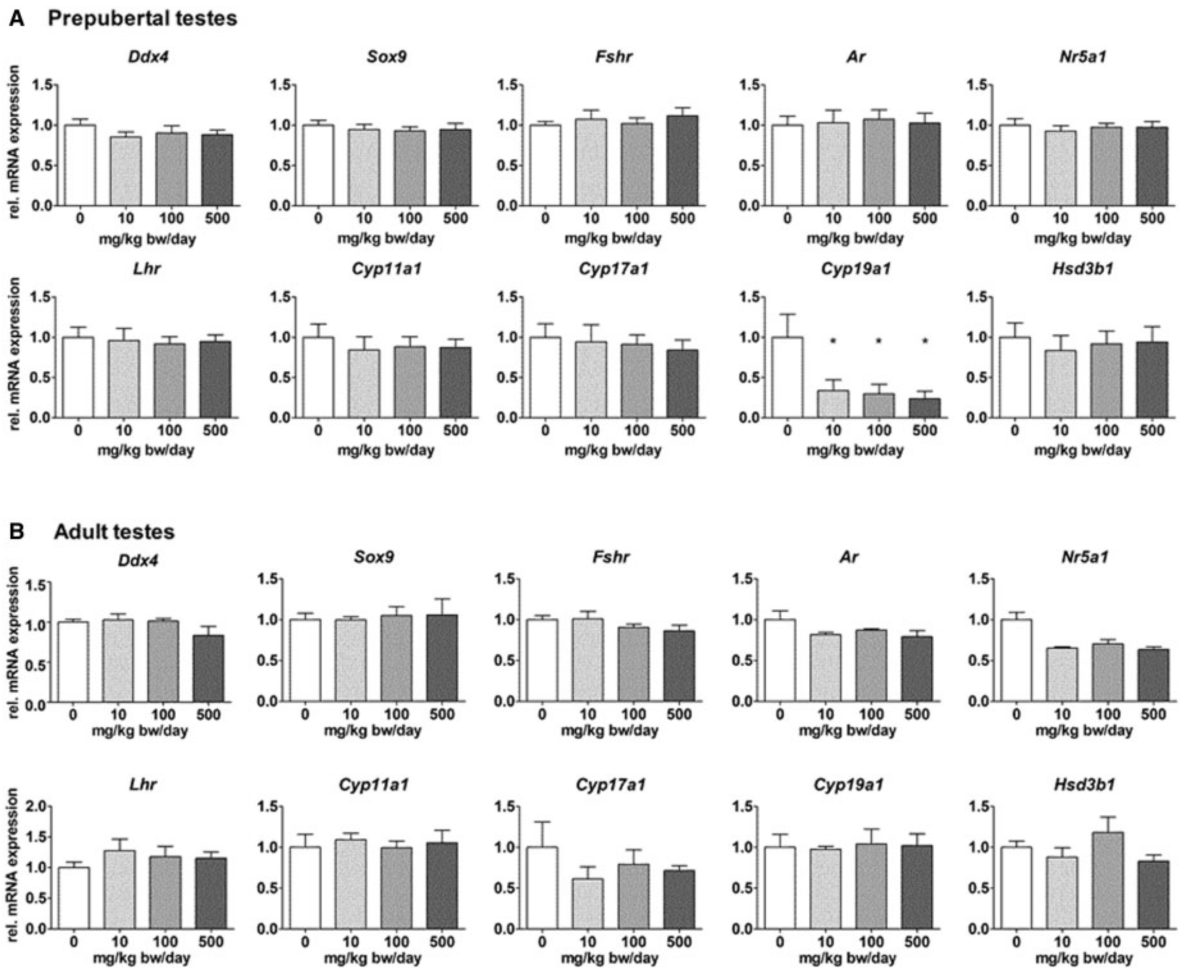

FIG. 3. Relative gene expression levels in PD16 and PD 90 testes from control and butylparabene-exposed rats. **A**, At PD 16, relative expression levels of the germ cell marker *Ddx4*, Sertoli cell markers *Sox9* and *Fshr*, the Sertoli/Leydig expressed genes *Ar* and *Nr5a1, and* Leydig cell markers *Lhr*, *Cyp11a1*, *Cyp17a1*, and *Hsd3b11* were unchanged following exposure to 10, 100, or 500 mg/kg bw/d butylparaben. Expression of *Cyp19a1* (aromatase) was significantly lower in testes from all exposure groups at PD 16. **B,** In adult testes, the expression of the same genes was unchanged except for *Nr5a1*, which was lower in testes from all exposed groups compared with controls. Relative expression levels are shown with mean value of control testes set to 1, with data normalized using the geometric mean of *Rpl13a* and *Sdha* (prepubertal, n = 9–10; adult, n = 4; Mean ± SEM; * $P < .05$).

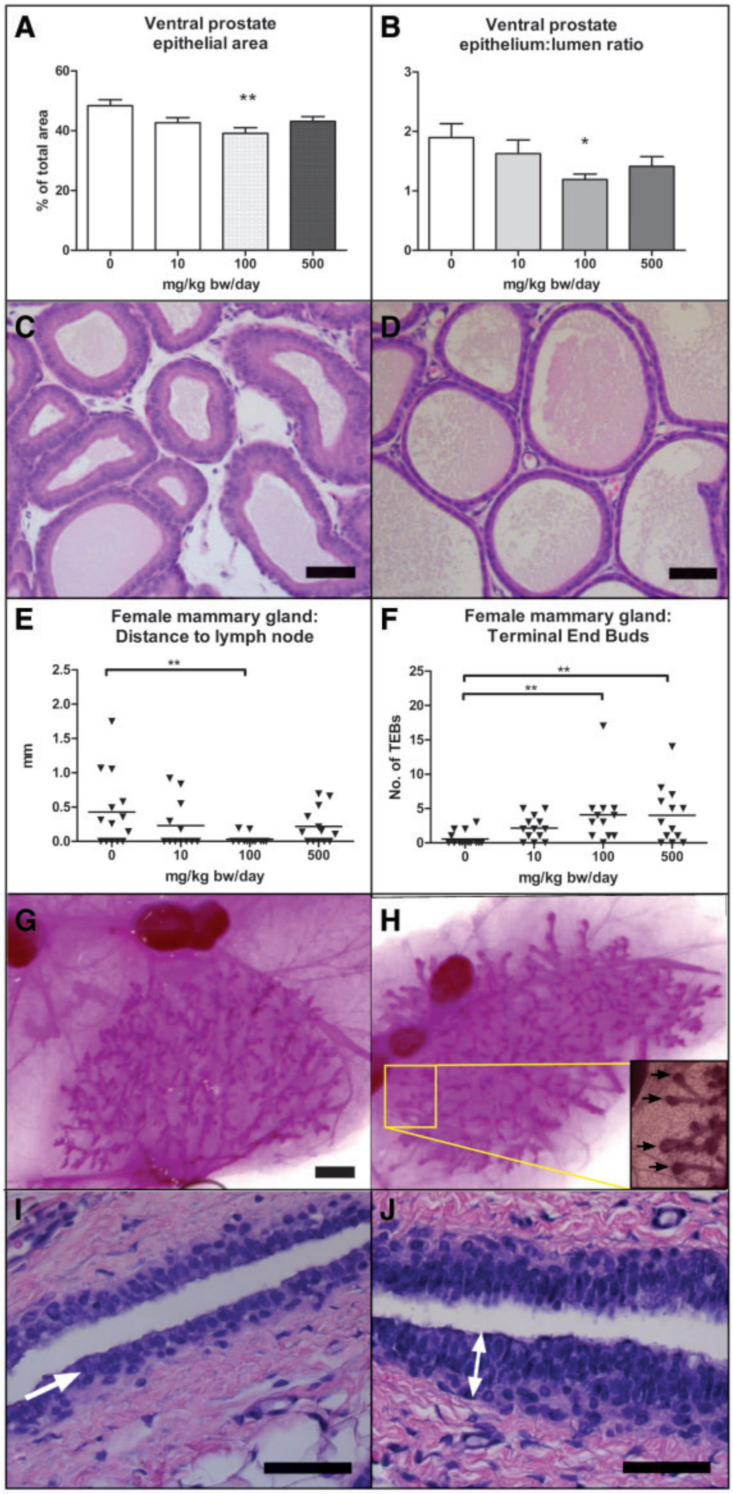

FIG. 4. Effects of perinatal butylparaben exposure of rats on early prostate and mammary gland development. **A and B,** Morphometric analysis revealed a reduced epithelial area (as a percentage of total prostate area) and reduced epithelium to lumen ratio in PD 22 male offspring from the middle dose group. Data represent group means +SEM, n = 11–14. Asterisks indicate statistically significant differences from controls analyzed in ANOVA followed by Dunnett's test, * $P < .05$; ** $P < .01$. Ventral prostate histology at PD 22, bars indicate 50 μm. Control animals **(C)** had columnar epithelium in ventral prostate, whereas enlarged acini with reduced epithelial height were seen mainly in the middle dose group **(D)**. **E,** Outgrowth of female mammary glands, measured as the distance from the mammary gland to nearest lymph node, was increased in animals exposed to 100 mg/kg bw/d butylparaben. **F,** The number of terminal end buds (TEBs) in females was increased at 100 and 500 mg/kg bw/d. Data points indicate measurements from individual animals with lines representing group means. ** indicate a statistically significant difference from controls with $p < 0.01$ analyzed by Kruskal-Wallis' nonparametric test followed by Dunn's posttest for multiple comparisons, n = 12–14. **G,** Representative mammary whole mount image of a PD 22 control female mammary gland. **H,** Representative mammary whole mount image of a PD 22 high-dose female mammary gland with an increased number of TEBs (black arrows). **I,** Mammary duct from a PD 90 control female. **J,** Intraductal hyperplasia (double-headed arrow) in mammary gland from a PD 90 female from the low-dose group.

TABLE 3. Comparison of Studies Examining Effects of Perinatal Exposure to Butyl- or Isobutyl Paraben in Rats

Chemical	Study Design	Effects on Bw, AGD, Sperm Count, Sperm Motility, Pubertal Onset	Remarks and Other Effects	References
Butylparaben	Subcutaneous exposure of Sprague Dawley rats from GD 6 to PND 20. 0, 100, or 200 mg/kg bw/d. Vehicle: DMSO. n = 6 control litters, 8 BP litters. 5–7 per sex per litter killed at PND 21, 49, 70, and 90.	From 100 mg/kg: Reduced female pup bw at PND 49, 70, and 90. Reduced epididymal sperm count and sperm motility at PND 90 (n = 5). No effect on male or female AGD at PND 1 (0.05 mm accuracy). Early VO at 100 mg/kg only. No data on age at PPS. No NOAEL was obtained.	Reduction of sperm count to ≈50% of controls; same effect size at both doses. Reduced number of live pups from 100 mg/kg. Reduced pup survival to weaning at 200 mg/kg. Decreased weight of testes, seminal vesicles and prostate in 100 mg/kg dose group at some ages only.	Kang *et al.* (2002)
Butylparaben	Oral exposure of Wistar rats from GD 7 to PND 21. 0, 64, 160, 400, or 1000 mg/kg bw/d. Vehicle: Corn oil. n = 7–8 litters. 7–8 males per dose group were killed at PND 21, 35, 49, 90, and 180.	From 400 mg/kg: Reduced male pup bw at several ages. Shortened male AGD on PND 1 and 21. Delayed PPS. Reduced epididymal sperm count and daily sperm production. No examination of sperm motility. NOAEL 160 mg/kg.	Slight reduction in sperm count at 160 mg/kg, but same effect size (reduction to 64% of controls) at 160 and 400 mg/kg. Male serum hormone levels: Decreased testosterone, increased estradiol and progesterone, and increased/ decreased LH and FSH depending on age and dose level starting from 400 mg/kg.	Zhang *et al.* (2014)
Isobutylparaben	Oral exposure of Sprague Dawley rats from GD 6 to PND 21. 0 or 2.5 mg/kg bw/d (in a study including also bisphenol A). Vehicle: corn oil. n = 3 litters. 8 males per dose groups (representing 3 litters) were killed at PND 70.	At 2.5 mg/kg: Reduced epididymal sperm count and sperm motility (n = 5). No change in male pup bw, male AGD or age at PPS. No NOAEL was obtained.	Small study, one dose of isobutylparaben only. Reduction of sperm count to ≈40% of controls. Reduced male serum estradiol at PND 70. No changes in testis or epididymis weight at PND 70.	Yang *et al.* (2015)
Butylparaben	Oral exposure of Wistar rats from GD 7 to PND 21. 0, 10, 100 or 500 mg/kg bw/d. Vehicle: corn oil. N = 18 litters. One male and one female pup per litter killed at each of 3 age groups: PND 16-17, 22, and 80-90.	From 10 mg/kg: Reduced epididymal sperm count. From 100 mg/kg: Shortened male and female AGD. No effect on pup bw, age at PPS or age at VO. No examination of sperm motility. No NOAEL was obtained.	Reduction of sperm count to 76–78% of controls; same effect size at all doses. Reduced ovary weight at PND 17 from 100 mg/kg, reduced prostate weight at PND 90 at 500 mg/kg, altered mammary gland development from 100 mg/kg. No effect on nipple retention of males.	Current study

AGD, anogenital distance; GD, gestation day; PND, postnatal day; NOAEL, no-observed adverse effect level.

subcutaneous exposure of dams to 100 and 200 mg/kg bw/d of butylparaben (Kang *et al.*, 2002), or oral exposure to 400 and 1000 mg/kg bw/d (Zhang *et al.*, 2014). Table 3 summarizes findings in rat studies with perinatal exposure to butylparaben or isobutylparaben. Zhang *et al.* (2014) did not see any changes in sperm counts at the dose levels applied in this study, although rat strain and study design were comparable. To some extent, our findings of effects at relatively low oral doses are supported by a study showing reduced sperm count and motility in rats perinatally exposed to 2.5 mg/kg bw/d of the structurally related isobutylparaben (Yang *et al.*, 2015).

In this study, a shortened neonatal male AGD was seen from 100 mg/kg bw/d, but the same was only seen at doses from 400 mg/kg bw/d of butylparaben in the study by Zhang *et al.* (2014), and not in the study by Kang *et al.* (2002) (see Table 3).In a previous study, we showed that subcutaneous exposure of pregnant dams to 200 or 400 mg/kg bw/d of butylparaben did not affect AGD or testicular testosterone synthesis in fetal male rats (Taxvig *et al.*, 2008). Shortened male AGD is often considered an indicator of antiandrogenicity, fitting with the known antiandrogenic effects of butylparaben *in vitro* (Chen *et al.*, 2007; Satoh *et al.*, 2005). Notably, this study showed a shallow dose–response relationship between butylparaben exposure and male AGD with a maximum decrease of only 7%. A comparably shallow dose–response curve was seen in a recent study on bisphenol A (Christiansen *et al.*, 2014). In contrast, no change in male AGD was seen after perinatal exposure to the potent estrogen ethinyl estradiol (Mandrup *et al.*, 2013). These findings may suggest that some chemicals with a common profile of estrogenic and weakly antiandrogenic effects only induce subtle effects on AGD.

To gain further insight into potential mechanisms behind the effects on male reproductive development, we analyzed the expression of several genes in prepubertal and adult testes. Several cell-specific marker genes were unchanged both at PD16 and adulthood, suggesting that the general testis cellularity was maintained. However, at PD16 the expression of *Cyp19a1* (aromatase) was lower in all dose groups compared with controls. CYP19 catalyzes the conversion of androgens into estrogens (Simpson *et al.*, 2002), and at this stage aromatase is expressed by immature rat Sertoli cells and is stimulated by FSH to elevate serum estradiol for a few days (Picut *et al.*, 2015). The role of estradiol at this age is not clear, but the increase in estradiol coincides with the first wave of spermatogenesis and establishment of the blood-testis barrier. Therefore, it may be speculated that the reduction of aromatase levels at this stage is related to the observed low sperm count later in life. Alternatively, our findings may reflect a slight delay in development of the exposed rats. Although butylparaben did not appear to affect estradiol synthesis or secretion at this age, it would be interesting to further scrutinize possible effects of butylparaben on testicular aromatase levels and estradiol production.

In adult testes we observed a reduction in *Nr5a1* expression in exposed animals. *Nr5a1* encodes a nuclear receptor commonly called Steroidogenic Factor-1 (SF-1), which is involved in the regulation of numerous genes, including several SFs (Buaas *et al.*, 2012). Thus, reduced NR5A1 could help explain alterations in hormone levels observed in adulthood in the study by Zhang *et al.* (2014). Actually, albeit not statistically significant, *Cyp17a1* showed a slightly lower expression in the same testes displaying reduced *Nr5a1* levels, which is in line with the known involvement of NR5A1 in the transcriptional regulation of several CYP genes, including *Cyp17*. Therefore, butylparaben exposure could be suggested to compromise reproductive function by interfering with steroidogenesis. However, butylparaben had no or limited effects on estradiol or testosterone levels adrenal H295R cells (Taxvig *et al.*, 2008).

The observed reduction in prostate and seminal vesicle weight in adult animals may also be a consequence of altered hormone production early or late in life. Indications of early changes in prostate development were seen as the relative epithelial area in PD 16 prostates was reduced at 100 mg/kg bw/d compared with controls, but we found no histological changes in prostates of young adults after butylparaben exposure. It is well known that developmental exposure to other estrogenic chemicals or elevated perinatal levels of estrogens in rodents can induce atypical hyperplasia of the prostatic epithelium (PIN lesions) and morphological changes resembling those of precancerous lesions in humans (reviewed by Prins *et al.*, 2007). Focal proliferative effects are, however, unlikely to be reflected by prostate weights. But neonatal exposure to high doses of estradiol benzoate in rats was found to induce prostatic PIN lesions and reduced dorsal prostate weight later in life (Ho *et al.*, 2006). Therefore, despite being indicative, our data on prostate weights alone do not allow us to conclude whether proliferative effects were induced by butylparaben or not, nor does it lend enough insight to elucidate possible effects of parabens on prostate cancer susceptibility. For this, further examination in older animals or in a cancer induction model is necessary.

Altered Female Reproductive Development

The fact that prepubertal female rats exposed to butylparaben displayed increased outgrowth of mammary glands and a greater number of TEBs supports an estrogenic mode of action. Similarly, an increase in mammary gland outgrowth in female rats has been seen with perinatal exposure to ethinyl estradiol or a mixture of estrogenic chemicals (Mandrup *et al.*, 2012, 2015), and with prepubertal exposure to the phytoestrogen genistein (Murill *et al.*, 1996). Early postnatal exposure to phytoestrogens or bisphenol A can increase the number of TEBs in prepubertal rats (Moral *et al.*, 2008; Tan *et al.*, 2004). In our previous study on ethinyl estradiol, we observed no change in TEB numbers in females, but an increase in male TEB numbers (Mandrup *et al.*, 2012). TEBs are the site of origin of mammary carcinomas and a target structure of carcinogens (Russo *et al.*, 1979; Russo and Russo, 1996b), and a link between chemically induced increases in TEB numbers and an increased risk of mammary cancer has been proposed (Fenton, 2006). In adult mammary gland, possible effects on intraductal hyperplasia and reduced lobular development were observed at 10 mg/kg bw/d, but the low incidence of intraductal hyperplasia and the lack of effect at higher doses complicate the interpretation of these findings. Interestingly, intraductal hyperplasia has also been observed at low doses of bisphenol A (Durando *et al.*, 2007; Murray *et al.*, 2007), emphasizing that some endocrine disruptors may induce proliferative changes in the mammary ducts at low, but not high doses (Mandrup *et al.*, 2016; Murray *et al.*, 2007; Vandenberg *et al.*, 2013). It may be speculated that the observed changes in early mammary development may be related to adverse effects later in life, but further studies are needed to clarify this.

The observed reduction in prepubertal ovary weight is in agreement with a study on neonatal rats showing delayed follicular recruitment at PD 8 after 7 days of subcutaneous exposure to propyl- and butylparaben (Ahn *et al.*, 2012). In our study, adult ovaries appeared normal, but it may be speculated that a possible interference with early follicle recruitment may have long-term adverse effects manifesting as altered reproductive function later in life. Currently, few studies have targeted the influence of chemicals on early ovarian development. One example of altered early ovary development with late-life consequences following exposure to a weak estrogen is the finding of inhibited germ cell nest breakdown on PND 4 and an early decline in fertility in mice prenatally exposed to low doses of bisphenol A (Wang *et al.*, 2014).

Few studies have reported changes in female AGD at birth, but we observed a slight reduction in female pup AGD (corrected for bw changes) following butylparaben exposure in this study and with bisphenol A exposure in a previous study (Christiansen *et al.*, 2014). In contrast, ethinyl estradiol increased the AGD and the number of retained nipples of female offspring (Mandrup *et al.*, 2013). Moreover, prochloraz exposure in utero increased female AGD in several studies (Laier *et al.*, 2006; Melching-Kollmuss *et al.*, 2016). Different effect patterns thus appear for weak and strong estrogens, but little is known regarding the relevance of these findings and whether such disruptions to endocrine homeostasis can predict any late life reproductive dysgenesis in females.

Implications for Risk Assessment

We found that both prepubertal aromatase expression and adult sperm count were affected at the lowest administered dose of butylparaben. This corresponds to the dose level previously shown to affect sperm count following juvenile dietary exposure in rats (Oishi, 2001), but disagreeing with 2 other studies on butyl- and propylparaben, respectively (Gazin *et al.*, 2013; Hoberman *et al.*, 2008). Due to these discrepancies, it has been difficult to determine a robust no-adverse effect level (NOAEL) for risk assessment. The Scientific Committee of Consumer Safety applied a no-effect level (NOEL) of 2 mg/kg bw/d in their risk assessment of butylparaben (SCCS 2013). This was based on the lack of effect on epididymis following juvenile subcutaneous exposure (Fisher *et al.*, 1999). In their opinion, SCCS considered that this starting point for risk assessment was a 'conservative choice'. However, the current data suggest that a NOAEL of 2 mg/kg bw/d is not conservative, as the observed reductions of epididymal sperm count and testicular aromatase expression at 10 mg/kg bw/d points to a NOAEL at or possibly below 2 mg/kg bw/d. This is further corroborated by the recent finding of reduced sperm count and motility in rats perinatally exposed to 2.5 mg/kg bw/d of the structurally related isobutylparaben (Yang *et al.*, 2015).

The SCCS discussed that rats may be a poor model for assessing and extrapolating potential harmful effects of butylparaben in humans, as rats have a much greater metabolic rate. For instance, internal exposure levels of free parabens measured in human males exposed dermally to 10 mg/kg bw/d were similar to internal exposure of rats exposed orally to 1000 mg/kg bw/d (Appendix 2 of SCCS 2013). Therefore, the SCCS noted that when using toxicokinetic data, it is uncertain whether the desired margin of safety of 25 can be achieved and thus, that uncertainties with risk assessment remain which presently cannot be resolved (SCCS 2013). Notably, blood levels of parabens do not directly reflect levels in solid tissues. Examination of tissue distribution of C14-labeled butylparaben and metabolites in rats showed higher tissue concentrations than in blood for oral, intravenous, as well as dermal exposure routes (Mathews *et al.*, 2013). Furthermore, fetal exposure levels may exceed maternal blood levels (Frederiksen *et al.*, 2008). Therefore, a concern remains that the developing human may not be sufficiently protected against some of the multiple endocrine disrupting effects of butylparaben, either alone or in combination with other endocrine disrupting chemicals.

In summary, butylparaben altered AGD, ovary and prostate weights, sperm counts, and mammary development, and thus exerted endocrine disrupting effects on both male and female offspring. Interestingly, gene expression profiling of testis indicated that some of the male reproductive effects may be related to changes in steroidogenesis.

ACKNOWLEDGMENTS

The presented research was made possible with contributions of laboratory technicians and assistants of whom we wish to thank for their excellent technical assistance Sarah Grundt Simonsen, Ulla El-Baroudy, Vibeke Kjær, Heidi Letting, Lillian Sztuk, Dorte Lykkegaard Korsbech, Birgitte Plesning, Bo Oscar Herbst, Gülcan Geyik, and Anne Ørngreen and Co-workers from the animal facility.

FUNDING

This study was part of a large project funded by the Centre on Endocrine Disrupters in Denmark and the Danish Environmental Protection Agency.

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