Prenatal Exposures of Male Rats to the Environmental Chemicals Bisphenol A and Di(2-Ethylhexyl) Phthalate Impact the Sexual Differentiation Process

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The increasing incidence of reproductive anomalies, described as testicular dysgenesis syndrome, is thought to be related to the exposure of the population to chemicals in the environment. Bisphenol A (BPA) and di(2-ethylhexyl)phthalate (DEHP), which have hormonal and antihormonal activity, have attracted public attention due to their presence in consumer products. The present study investigated the effects of BPA and DEHP on reproductive development. Timed-pregnant female rats were exposed to BPA and DEHP by gavage from gestational days 12 to 21. Results showed that prenatal exposures to test chemicals exerted variable effects on steroidogenic factor 1 and GATA binding protein 4 protein expression and increased ($P < .05$) sex-determining region Y-box 9 and antimüllerian hormone protein in the infantile rat testis compared with levels in the control unexposed animals. Pituitary LHβ and FSHβ subunit protein expression was increased ($P < .05$) in BPA- and DEHP-exposed prepubertal male rats but were decreased ($P < .05$) in adult animals relative to control. Exposure to both BPA and DEHP in utero inhibited ($P < .05$) global DNA hydroxymethylation in the adult testis in association with altered DNA methyltransferase protein expression. Together the present data suggest that altered developmental programming in the testes associated with chemical exposures are related to the disruption of sexual differentiation events and DNA methylation patterns. The chemical-induced effects impact the development of steroidogenic capacity in the adult testis. **(*Endocrinology* 156: 4672–4683, 2015)**

Environmental chemicals that exert hormonal effects and interfere with the endocrine axis are described as endocrine-disrupting chemicals (EDCs) (1). EDCs are thought to act primarily through nuclear hormone receptors, including estrogen receptors (ESRs), androgen receptors (ARs), progesterone receptors, and thyroid hormone receptors. However, recent studies indicated that EDCs may also act through nonnuclear steroid hormone receptors (eg, membrane ESRs) and nonsteroidal receptors (eg, neurotransmitter and orphan receptors) (1). Several EDCs used in industrial and manufacturing activities are found in the environment, including bisphenol A (BPA), polychlorinated biphenyls, pesticides, phthalates, polybrominated diphenyl ethers, and heavy chemicals, eg, cadmium chloride.

BPA is widely used in the manufacture of polycarbonate plastics (eg, water bottles, baby bottles) and epoxy resins to line the inside of metallic food cans and as a nonpolymer additive to other plastics (2, 3). Mean urinary levels of BPA in the adult US population was estimated at approximately 2.5 ng/mL (4). Similarly, phthalate esters have attracted considerable attention due to their high

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Abbreviations: ACTB, β-actin; AMH, antimüllerian hormone; AR, androgen receptor; BPA, bisphenol A; bw, body weight; DEHP, di(2-ethylhexyl)phthalate; dG, 2′-deoxyguanosine; Dnmt, DNA methyltransferase; E2, 17β-estradiol; EDC, endocrine-disrupting chemical; ESR, estrogen receptor; GATA-4, GATA binding protein 4; GD, gestational day; 5hmc, 5-hydroxymethyl-2′-deoxycytidine; Insl3, insulin-like factor 3; 5mc, 5-methyl-2′-deoxycytidine; PND, postnatal day; SF1, steroidogenic factor 1; Sox9, sex-determining region Y-box 9.

production volume and use in a variety of polyvinyl chloride-based consumer products. As a constituent of infant toys, building and food packaging products, and biomedical devices, di(2-ethylhexyl)phthalate (DEHP) is the most abundant phthalate in the environment (5), with daily exposure levels estimated at approximately 30 mg/kg body weight (bw) (6). Although epidemiological data are scanty, there is considerable public concern that prenatal exposure of the developing male to EDCs may have adverse effects on the masculinization process (7, 8). For example, greater phthalate concentrations were measured in cryptorchid than in noncryptorchid babies (9), whereas serum phthalate concentrations were positively correlated with the incidence of cryptorchidism and shortened anogenital distance in male infants (10, 11).

Once gonadal sex has been determined, sexual differentiation in the male is regulated largely by the antimüllerian hormone (AMH) and androgens produced by the fetal testis (7). The AMH is a glycoprotein secreted by Sertoli cells that induces the regression of Mullerian ducts during development (12). AMH is considered to be a marker for prepubertal Sertoli cell development because its expression decreases as Sertoli cells mature. Androgens are produced by fetal Leydig cells to support reproductive tract development (13, 14). The primary male sex pathway is initiated by the sex-determining region Y (*SRY*) gene, which stimulates AMH secretion by Sertoli cells (15, 16). Downstream of the *SRY* gene, many transcription factors, including sex-determining region Y-box 9 (Sox9), steroidogenic factor 1 (SF1) and GATA binding protein 4 (GATA-4), regulate sexual differentiation by their action on AMH (17). Interestingly, we observed previously that BPA regulated expression of the AMH peptide in prepubertal Sertoli cells (18). SF1 also acts as the major transcriptional regulator of insulin-like factor 3 (Insl3) and steroidogenic enzymes that control androgen production in Leydig cells (19, 20). In addition, we have demonstrated that perinatal exposures of male rats to BPA and DEHP stimulated proliferative activity in testicular Leydig cells early in development and affected androgen secretion in the adult testis (21, 22). Other reports showed that developmental exposures of male rats to BPA and DEHP disrupted the blood-testis barrier and caused testicular germ cell depletion (23, 24). However, the molecular basis for chemical-induced disruption of testicular development is not fully understood.

We hereby propose that the adult testis effects related to developmental chemical exposures result from hormonal and antihormonal activity that interfere with the sexual differentiation process. For example, the biological actions of BPA are thought to be due to its estrogenic properties (25), whereas DEHP is considered to be a classical antiandrogen (26). This classification is possibly misleading because we and others have shown that BPA has the capacity to block AR-mediated transcriptional activity (27), whereas exposure to DEHP induced aromatase activity and increased estrogen biosynthesis in the testis (22, 28). Accordingly, the present study used a comparative approach to study aspects of hormonal and genetic activity of BPA and DEHP that regulate the endocrine axis and affect development of steroid hormone secretion capacity in the testis. We analyzed gene expression in the infantile and adult testis, gonadotropin subunit (lutropin subunit-β [LHβ] and follitropin subunit-β [FSHβ]) expression in the pituitary glands of prepubertal and adult male rats and measured steroid hormone secretion in the adult testis. In addition, we explored the possibility that chemical exposure effects occurring early in development induced epigenetic changes (29, 30) by the analysis of global DNA methylation patterns in the adult testis and the expression of DNA methyltransferases (Dnmts) in Western blots.

Materials and Methods

All experimental and euthanasia procedures were performed in accordance with a protocol approved by the Auburn University Institutional Animal Care and Use Committee and the recommendations of the panel on Euthanasia of the American Veterinary Medical Association. Time-bred, pregnant Long-Evans dams at gestational day 6 were obtained from Harlan-Teklad and were allowed to acclimatize for 5–6 days at the College of Veterinary Medicine Division of the Laboratory Animal Health Housing Facility. Pregnant and nursing dams were housed one per cage, whereas weanling rats were kept in groups of two to four, depending on age and size. Animals were kept on a 12-hour light, 12-hour dark cycle with ambient temperature of 68°F-74°F and were provided feed and water ad libitum. It has been suggested that the prolonged use of polycarbonate cages may result in the leaching of BPA and other chemicals into the environment (29). Therefore, rats were housed in polypropylene cages with glass water bottles. Assignment of rats to groups was done by body weight randomization to ensure equal weight distribution.

Animal studies

The gestational day (GD) 12–21 time period is considered the critical time for male sexual differentiation in the rat under control of several transcription factors (30). To mitigate the potential confounding effects of physical handling associated with the activation of the hypothalamus-adrenal axis (31), we used two groups of control rats: control animals that did not receive the oil vehicle and a separate group that was gavaged only with the oil vehicle without BPA and DEHP. We also considered that altered cellular function occurring at low-dose exposures are more apt to result from receptor-ligand interactions than exposures occurring at high doses that cause cellular damage and overt toxicity. Therefore, a biologically based response model of low dose exposure paradigms was adopted. BPA was administered at 2.5 or 25 and DEHP at 5 or 50 μg/kg bw by oral gavage in the oil

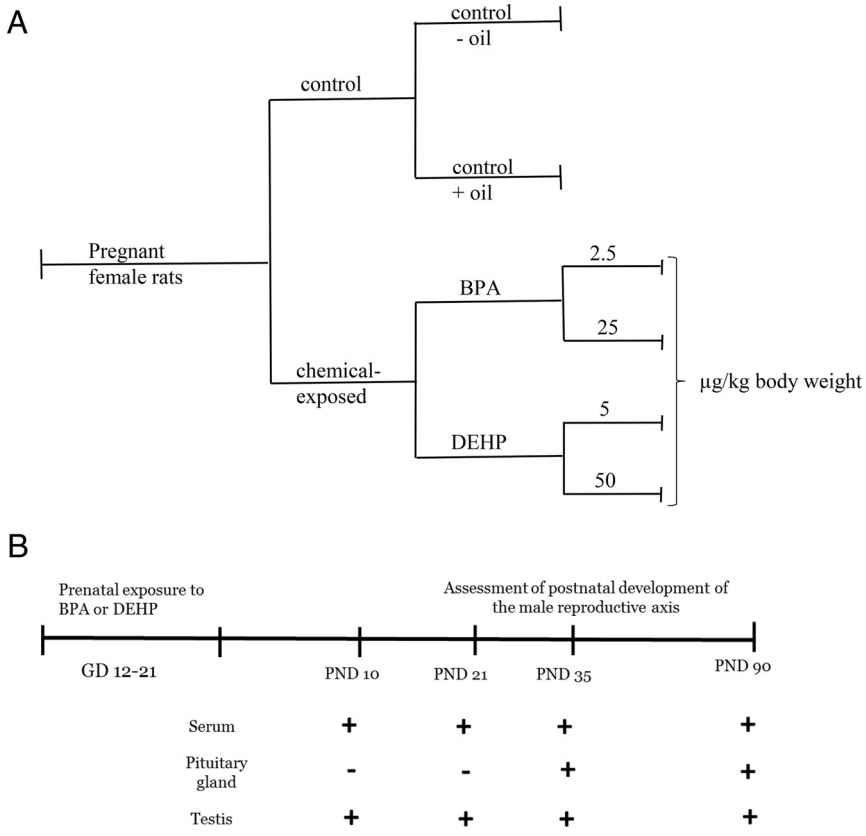

Figure 1. Schematic representation of the experimental protocol. A, Time-bred Long-Evans dams (8–11 female rats/group from two separate and independent experiments) served as control or were administered BPA or DEHP by gavage from GD 12 to GD 21, ie, six treatment groups. B, Cohorts of male rats from control and BPA- or DEHP-treated groups were chosen randomly from each litter and killed at 10, 21, 35, and 90 days of age to obtain serum, testes, and pituitary glands that were processed as described in *Materials and Methods*. Data are presented in Table 2 and Figures 2–6.

vehicle from GD 12 to parturition on GD 21. Altogether, timed-pregnant Long-Evans female rats (Harlan-Teklad) were grouped into six groups. Pregnant animals were weighed every other day, and the average body weight for each group was used to calculate chemical doses to administer. This experiment was performed on two separate occasions (n = 8–11 dams/group for combined experiments). After all dams carried pregnancy to full term, litter size, pup weight, and sex ratios were assessed on the day of birth, which was designated postnatal day (PND) 1. Male offspring were consequently and randomly obtained from every dam in each group and analyzed at 10, 21, 35, and 90 days postpartum (Figure 1). As required, testes were collected at the time the animals were killed, dissected free of the epididymis, and, with pituitary glands, stored at −80°C until the samples were homogenized and prepared for Western blotting analysis. To detect chemical-induced changes in hormone concentrations (ie, T, 17β-estradiol [E2], and AMH), trunk blood was collected at the time the animals were killed to obtain serum. Testicular tissue and purified Leydig cell fractions were processed to measure steroid hormone secretion ex vivo. In addition, testis tissue was processed to obtain genomic DNA for the assessment of global DNA methylation patterns.

Hormone measurements

Serum was separated from trunk blood collected from male rats at the time the animals were killed on PND 10, 21, 35, and 90. Testicular explants and aliquots of Leydig cells ($0.1–0.2 \times 10^6$) were incubated in microcentrifuge tubes. The culture medium consisted of DMEM/F12 buffered with 14 mm $NaHCO_3$ and 15 mm HEPES and contained 0.1% BSA and 0.5 mg/mL bovine lipoprotein. Incubation was conducted with a maximally stimulating dose of ovine LH (100 ng/mL) for 3 hours at 34°C. Steroid hormone concentrations (T, E2) were assayed in aliquots of spent media and serum samples using a tritium-based RIA with an inter-assay variation of 7%–8% (18). Hormone production was normalized to nanograms per testicular mass (milligrams) and 10^6 Leydig cells. The T to E2 ratios were calculated for each time point.

Isolation of Leydig cells

Leydig cells were isolated from the testis of 90-day-old Long-Evans male rats after they were killed by CO_2 asphyxiation using our standard procedures (18, 21, 22). Briefly, testes were collected and digested in dissociation buffer containing 0.25 mg/mL collagenase, 46 μg/mL dispase, and 6 μg/mL deoxyribonuclease for 1 hour in a shaking water bath at 34°C. Cellular suspension was subjected to centrifugation according to a procedure described previously (32). After centrifugation using a 55% continuous Percoll gradient, Leydig cells were isolated at the bottom of the tubes at a density equivalent to 1.070 or greater according to our standard procedure. The purity of Leydig cell fractions was assessed by histochemical staining for 3β-hydroxysteroid dehydrogenase using 0.4 mM etiocholan-3β-ol-17-one enzyme substrate (catalog number E-5251, lot number 11K4058; Sigma) (33). Cell numbers were estimated with a hemocytometer. Steroid hormone secretion by Leydig cells were assessed ex vivo by a RIA.

Gene expression analysis using SDS-PAGE and Western blot analyses

Expression of SF1, GATA-4, Sox9, AMH, Insl3, aromatase, AR, LHβ, FSβ, Dnmt3a and Dnmt3b protein in the serum, pituitary gland, and testis as appropriate at different ages was investigated by Western blot analysis. Tissues were homogenized in T-PER lysis buffer (Pierce Chemical Co) freshly supplemented with a protease inhibitor cocktail (catalog number 78410; Pierce Biotechnology, Inc). Tubes were centrifuged at 3000 rpm for 14 minutes at 4°C to remove cellular debris. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories) with BSA as standard. Aliquots (50 μL) of whole-cell lysate were dissolved in an equal volume of Laemmli buffer containing 5% β-mercaptoethanol and were boiled for 5 minutes at 95°C. To analyze serum AMH, serum protein concentrations were determined by the standard Bio-Rad assay. The serum sam-

ples were diluted in Laemmli buffer and then heated at 65°C for 10–20 minutes. All samples were resolved on varying percentages of Tris-HCl acrylamide gels by SDS-PAGE. Proteins were transferred to nitrocellulose membranes (catalog number 1620147; Bio-Rad Laboratories), which were subsequently incubated with blocking buffer (5% whole milk in 0.1% Tween 20 PBS) for 1 hour at room temperature to reduce nonspecific binding by antibodies. Membranes were then incubated in blocking buffer containing appropriate primary antibodies overnight at 4°C. Parameters of primary antibodies used in the present study are provided in Table 1. On the next day, blots were washed three times in 0.1% Tween 20 PBS to remove unbound antibody before incubation with the appropriate horseradish peroxidase-conjugated secondary antibody. Afterward, membranes were washed four times with 0.1% Tween 20 PBS and incubated with a chemiluminescent developing reagent (catalog number E2400; Denville Scientific) for 1 minute before exposure to X-ray films (catalog number E-3012; Denville Scientific). The presence of the proteins of interest was visualized by developing the film and were then scanned using an Epson 4180 Perfection scanner (Epson-America). The relative protein amounts in the identified immunoblots were measured as an OD of the bands on the exposed autoradiographic films using Doc-lt LS software (Ultra-Violet Products Ltd). Proteins were normalized to β-actin (ACTB).

Global DNA methylation analysis

Regarding assays designed to assess the potential for BPA and DEHP to induce epigenetic changes, we used the whole testis to preserve testis histoarchitecture that approximates the in situ paracrine relationship between testicular cells. Genomic DNA was extracted from testis of adult male rats at 90 days of age using the ZR Genomic DNA-Tissue MiniPrep kit in accordance with the protocol of the manufacturer (Zymo Research Corp). An selected reaction monitoring-based mass spectrometry assay was used to quantify 5-hydroxymethyl-2′-deoxycytidine (5hmc) and 5-methyl-2′-deoxycytidine (5mc). The assay was designed to measure 5HmdC (5-hydroxymethyl-2′-deoxycytidine) concentrations and 5mdC (5-methyl-2′-deoxycitidine) concentrations as a percentage of 2′-deoxyguanosine (dG) (eg, [5hmc]/[dG] and [5mc]/[dG]). The calibrated ranges for the analytes were 0%–2.5% for 5hmc and 0%–25% for 5mc using a fixed 40 pmol amount of dG as an internal standard. The samples had a measured range of 5hmc as low as 0.033% and as high as 0.057%. The samples had a measured range of 5mc between 4.081% and 4.834%. Replicates for the unknown samples were run in triplicate followed by a blank to eliminate carryover into the next unknown run (Supplemental Figure 1). All the procedures were carried out by Zymo Research Epigenetic Services (Zymo Research Corp).

Table 1. Antibody Table

Target	Antigen Sequence	Name of Antibody	Manufacturer, Catalog Number	Monoclonal or Polyclonal	Dilution Used
SF1	Belongs to the nuclear hormone receptor family. NR5 subfamily; contains one nuclear receptor DNA-binding domain	Anti-SF1 antibody (EPR11695 [B])	Abcam, Ab168380 MW (SF1) 52 kDa	Rabbit monoclonal IgG	0.0010
GATA-4	Genetic locus: GATA4 (human) mapping to 8p23.1; Gata4 (mouse) mapping to 14 D1	GATA-4 (C-20)	Santa Cruz Biotechnologies, Sc-1237 MW (GATA-4) 45 kDa	Goat polyclonal IgG	0.0020
AMH	Genetic locus: AMH (human) mapping to 19p13.3; AMH (mouse) mapping to 10C1	MIS (C-20)	Santa Cruz Biotechnologies, Sc-6886 MW (AMH) 70/74 kDa	Goat polyclonal IgG	0.0020
Sox9	Synthetic peptide from human Sox9	Anti-Sox9	Millipore, AB5535 MW (Sox9) 65 kDa	Rabbit polyclonal IgG	0.0002
ESR1	Belongs to the nuclear hormone receptor family; NR3 subfamily; contains one nuclear receptor DNA-binding domain	Anti-ESR-α (33) antibody-ChIP grade	Abcam, Ab2746 MW (ESRα) 68 kDa	Mouse monoclonal IgG	0.0001
ESR2	Belongs to the nuclear hormone receptor family; NR3 subfamily; contains one nuclear receptor DNA-binding domain	Anti-ESR-β antibody (9.88)	Abcam, Ab16813 MW (ESRβ) 59 kDa	Mouse monoclonal IgG	0.0001
AR	Belongs to the nuclear hormone receptor family. NR3 subfamily; contains one nuclear receptor DNA-binding domain	Anti-AR antibody (EP670Y)	Abcam, Ab52615 MW (AR) 99 kDa	Rabbit monoclonal IgG	0.0010
LHβ	Genetic locus: LHB (human) mapping to 19q13.33; Lhb (mouse) mapping to 7B4	Lutropin-β (R-16)	Santa Cruz Biotechnologies, Sc-7824 MW (LHβ) 22 kDa	Goat polyclonal antibody IgG	0.0010
FSH β	Genetic locus: FSHB (human) mapping to 11q14.1; Fshb (mouse) mapping to 2E3	FSHβ (C-19)	Santa Cruz Biotechnologies, Sc-7797 MW (FSHβ) 24 kDa	Goat polyclonal IgG	0.0005
CYP 19	Genetic locus: CYP19A1 (human) mapping to 15q21.2; Cup19a1 (mouse) mapping to 9A5.3	CYP 19 (H-300)	Santa Cruz Biotechnologies, Sc-30086 MW (CYP 19) 50 kDa	Rabbit polyclonal IgG	0.0010
Dnmt3a	Genetic locus: Dnmt3a (human) mapping to 2p23.3; Dnmt3a (mouse) mapping to 12A1.1	Dnmt3a (C-12)	Santa Cruz Biotechnologies, Sc-365769 MW (Dnmt3a) 100–130 kDa	Mouse monoclonal IgG	0.0002
Dnmt3b	Belongs to C5-methyltransferase family; contains one ADD domain, one GATA-type zinc finger, 1PWWP domain	Anti-Dnmt3b antibody (EPR3523)	Abcam, Ab 79822 MW (AR) 96 kDa	Rabbit monoclonal IgG	0.0010
INSL3	Genetic locus: Insl3 (mouse) mapping to 8 B3.3	INSL3	Santa Cruz Biotechnologies, Sc-134587 MW (Dnmt3a) 100–130 kDa	Rabbit polyclonal antibody IgG	0.0010
Actin	Epitope mapping at the C\ terminus of actin of human origin	ACTIN (I-19)	Santa Cruz Biotechnologies, Sc-1616 MW (actin) 43 kDa	Goat polyclonal IgG	0.0005

Abbreviations: CYP 19 = aromatase; ESR1 = estrogen receptor 1; ESR2 = estrogen receptor 2; MW, molecular weight.

Table 2. Body and Testis Weights With Serum Steroid Hormone Measurements in Male Rats Exposed to BPA and DEHP in the Prenatal Period[a]

Parameters	Control 0	Dose of BPA, kg/bw · d		Dose of DEHP, kg/bw · d	
		2.5 μg	25 μg	5 μg	50 μg
Body weights, g, PND 1 (n =	6.97 ± 0.36	6.78 ± 0.37	6.72 ± 0.21	6.42 ± 0.41	6.76 ± 0.36
Body weights, g, PND 5	13.49 ± 1.78	11.90 ± 0.42	11.13 ± 0.91[a]	12.24 ± 0.73	12.71 ± 0.64
Body weights, g, PND 10	24.78 ± 0.95	23.21 ± 0.65	22.85 ± 0.72	22.46 ± 0.83	23.09 ± 0.88
Body weights, g, PND 21	47.05 ± 7.80	44.51 ± 3.65	41.25 ± 3.68	44.68 ± 2.42	44.28 ± 1.48
Paired testis weights, g, PND 21	0.22 ± 0.02	0.21 ± 0.03	0.19 ± 0.02	0.22 ± 0.01	0.21 ± 0.01
Serum T, PND 21	1.24 ± 0.20	1.13 ± 0.11	1.33 ± 0.14	1.26 ± 0.11	1.44 ± 0.08
Serum E2, PND 21	0.20 ± 0.02	0.19 ± 0.01	0.19 ± 0.01	0.18 ± 0.01	0.20 ± 0.01
Serum T to E2 ratio, PND 21	6.38 ± 1.14	5.84 ± 0.54	7.11 ± 0.79	7.20 ± 1.01	7.28 ± 0.56
Body weights, g, PND 35	112.45 ± 11.60	109.52 ± 10.40	104.63 ± 7.06	115.83 ± 9.50	113.85 ± 5.07
Paired testis weights, g, PND 35	1.162 ± 0.07	1.06 ± 0.05	0.94 ± 0.14[a]	1.07 ± 0.09	0.91 ± 0.16
Serum T, PND 35	1.27 ± 0.14	1.41 ± 0.50	0.47 ± 0.08[a]	0.57 ± 0.23[a]	0.58 ± 0.17[a]
Serum E2, PND 35	0.14 ± 0.01	0.15 ± 0.01	0.13 ± 0.01	0.14 ± 0.01	0.12 ± 0.01
Serum T to E2 ratio, PND 35	9.17 ± 1.82	6.28 ± 1.46	3.65 ± 0.84[a]	4.37 ± 2.33	4.25 ± 1.32
Body weights, g, PND 90	425.72 ± 15.9	450.75 ± 16.80	465.46 ± 9.74	451.3 ± 23.8	477.98 ± 11.57[a]
Paired testis weights, g, PND 90	3.54 ± 0.08	3.00 ± 0.74	3.47 ± 0.08	3.54 ± 0.11	3.67 ± 0.09
Serum T, PND 90	3.35 ± 0.52	5.82 ± 1.59	4.03 ± 0.63	5.29 ± 0.78	6.17 ± 1.39
Serum E2, PND 90	0.22 ± 0.01	0.20 ± 0.02	0.23 ± 0.01	0.25 ± 0.01	0.29 ± 0.01[a]
Serum T to E2 ratio, PND 90	16.04 ± 2.64	27.28 ± 6.19	18.29 ± 3.08	21.38 ± 3.21	23.16 ± 5.68

Cohorts of male offspring were randomly selected and measured and/or processed at PND 1, 5, 10, 21, 35, and 90.

[a] Pregnant dams were gavaged with oil vehicle, BPA, and DEHP from GD 12 to GD 21.

Statistical analysis

Measurements were obtained from the two separate and independent experiments. Data are presented as the mean ± SD. Data describing pregnancy outcome and reproductive parameters up to PND 5 were based on the litter as a unit of measurement, whereas the parameters obtained thereafter were collected from randomly selected male rats within each treatment group. Data were analyzed either by an Student's unpaired *t* test for two groups or one-way ANOVA followed by the Dunnett's test for multiple groups comparison when there were more than two groups (Graph Pad Inc). Differences of $P < .05$ were considered to be significant.

Results

General observations after prenatal exposure of male rats to BPA and DEHP

We assessed whether the process of handling pregnant dams for gavage affected our end points of interest in the male offspring. Serum T with testicular T and E2 concentrations were similar ($P > .05$) in the two groups of control animals. However, serum E2 concentrations were decreased in control animals that were gavaged with the oil vehicle compared with the control animals that did not receive the oil vehicle ($P < .05$) (Supplemental Figure 2). Exposure of timed-pregnant dams to BPA and DEHP did not affect pregnancy outcome, eg, litter size and pup sex ratios (data not shown). Body weights of male rats measured at PND 1, 5, 10, 21, 35, and 90 were similar in treatment groups but were increased in the greater DEHP dosage group at 90 days of age compared with control animals ($P < .05$). On the other hand, paired testes weights were decreased ($P < .05$) in the greater BPA dosage group at 35 days of age but, otherwise, were similar in all other groups (Table 2).

Exposure to BPA and DEHP in utero affected gene expression in the infantile male rat testis

To identify chemical exposure effects on transcriptional regulation of the sexual differentiation process, we analyzed gene expression in the infantile testis. As described in Figure 2, expression of SF1 protein was decreased ($P < .05$) only in the testis of 10-day-old male rats exposed to the smaller but not greater BPA dose (Figure 2A) and was unaffected by DEHP at both doses (Figure 2B). GATA-4 protein was increased ($P < .05$) by exposure to the greater BPA dose (Figure 2C), whereas the two DEHP doses caused opposite effects whereby the smaller dose increased ($P = .05$), but the greater dose caused a decrease compared with the control ($P < .05$) (Figure 2D). There were dose-dependent increases ($P < .05$) in the expression of Sox9 and AMH protein after exposure to BPA and DEHP compared with the levels in unexposed control animals, but the smaller BPA dose did not affect AMH protein expression (Figure 2, E–H). A Western blot analysis of testicular Insl3 protein did not show significant treatment effects in BPA- and DEHP-exposed male rats versus control at 35 days of age (data not shown).

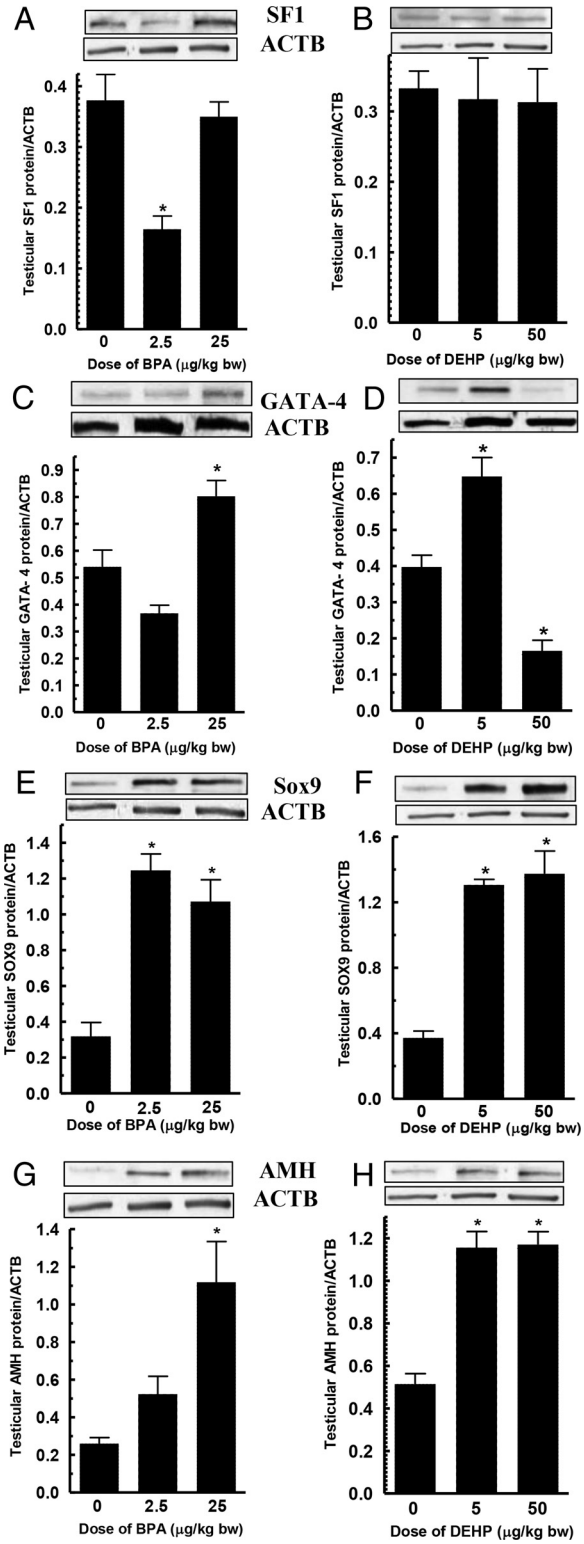

Figure 2. Male rats were exposed to BPA and DEHP by maternal gavage from GD 12 to GD 21. Testicular tissue was obtained from infantile male rats at 10 days of age (n = 6/group) to analyze SF1 (A and B), GATA-4 (C and D), Sox 9 (E and F), and AMH (G and H). Protein levels were measured using Western blotting procedures and the appropriate primary and secondary antibodies. Assays per sample were repeated at least four times and protein levels were normalized to ACTB. *, $P < .05$ vs control.

Prenatal exposure to BPA and DEHP altered gonadotropin subunit protein expression in pituitary glands of prepubertal and adult male rats

We evaluated the chemical exposure effects on gonadotropin subunit expression during development. In prepubertal male rats (PND 35), pituitary LHβ-subunit protein expression was increased ($P < .05$) after exposure to both BPA doses, but this effect was associated only with the greater DEHP dose (Figure 3, A and B). On the other hand, BPA exposure did not affect the expression of the FSHβ-subunit protein ($P > .05$), but the greater DEHP dose increased FSHβ protein in 35-day-old male rats (Figure 3, C and D). In sexually mature animals, ie, at 90 days of age, the expression of LHβ and FSHβ protein was decreased ($P < .05$) in all animals exposed to the greater BPA and DEHP doses compared with the control (Figure 3, E–H).

Gestational exposure to BPA and DEHP affected AMH, aromatase, and AR protein expression in adult male rats

The possibility that chemical exposures occurring in utero caused permanent effects that persisted into adulthood was investigated in a subset of end points. A Western blot analysis demonstrated that serum AMH protein was increased ($P < .05$) in the adult male rats after prenatal exposures to BPA and DEHP compared with the control (Figure 4, A and B). The increase in serum AMH protein was associated with increased ($P < .05$) aromatase and AR protein expression in the testis of adult animals (Figure 4, C–F).

Prenatal exposure to BPA and DEHP affected serum hormone concentrations and differentiation of steroidogenic capacity in the testis of male rats

To determine whether chemical exposure effects impacting the sexual differentiation process affected endocrine function of the testis later in life, we measured steroid hormone secretion in the adult testis. Serum T concentrations were decreased ($P < .05$) in the greater BPA as well as both DEHP dosage groups at 35 days but were similar in all groups at 21 and 90 days of age ($P > .05$) compared with the control. Serum E2 concentrations were increased ($P < .05$) in the greater DEHP dosage group at 90 days and were similar in other treatment and control groups at all ages. Serum T to E2 ratios were decreased ($P < .05$) in the greater BPA dosage group at 35 days compared with the control but were otherwise similar in other treatment groups (Table 2). Testicular T production, measured ex vivo, was not affected by exposure to BPA but was greater ($P < .05$) in the DEHP-treated male rats at 90 days of age

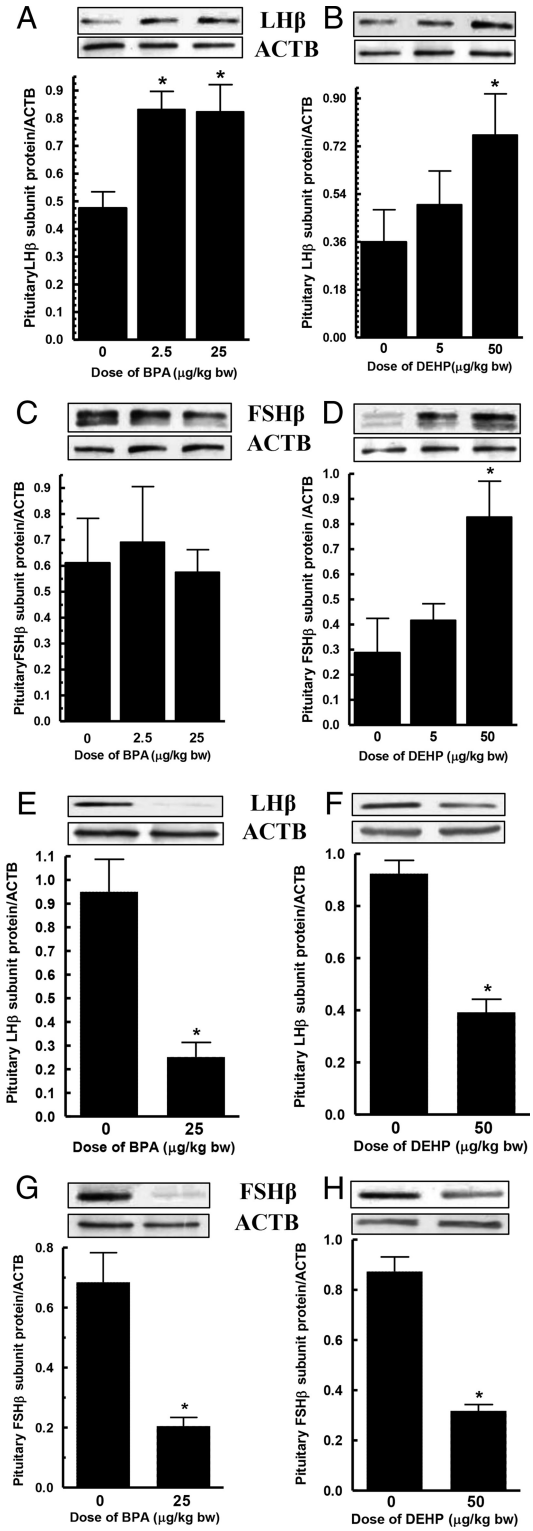

Figure 3. Male rats were exposed to BPA and DEHP by maternal gavage from GD 12 to GD 21. Gonadotropin subunit protein was analyzed in pituitary glands in prepubertal male rats at 35 days of age (n = 6 animals/group): LHβ (A and B) and FSHβ (C and D); and in 90-day-old male rats (n = 6 animals/group): LHβ (E and F) and FSHβ (G and H). Protein measurements were done by Western blotting procedures using antisera specific to rat LHβ and FSHβ and the appropriate secondary antibodies. Assays per sample were repeated at least four times and protein levels were normalized to ACTB. *, $P < .05$ vs control.

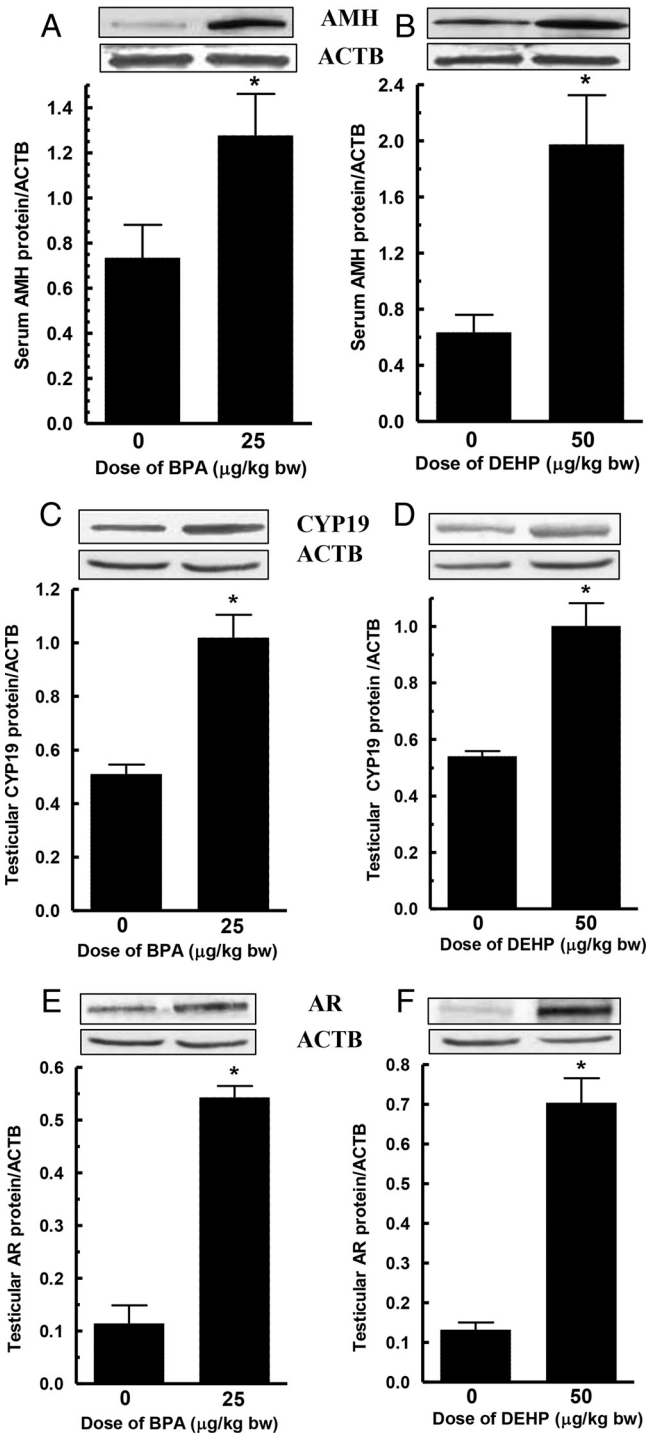

Figure 4. Male rats were exposed to BPA and DEHP by maternal gavage from GD 12 to GD 21. Adult male rats at 90 days of age were processed to obtain serum (n = 10 animals per group) to measure AMH protein (A and B) and testicular tissue (n = 6 animals per group) to analyze aromatase (Cyp19) (C and D) and AR protein (E and F). Protein analysis was achieved by Western blotting procedures using antisera to rat AMH, aromatase, and the AR. Assays per sample were repeated at least four times and protein levels were normalized to ACTB. *, $P < .05$ vs control.

compared with the control (Figure 5, A and B). In contrast, testicular E2 production was increased ($P < .05$) by exposure to both BPA doses, but this effect was caused only

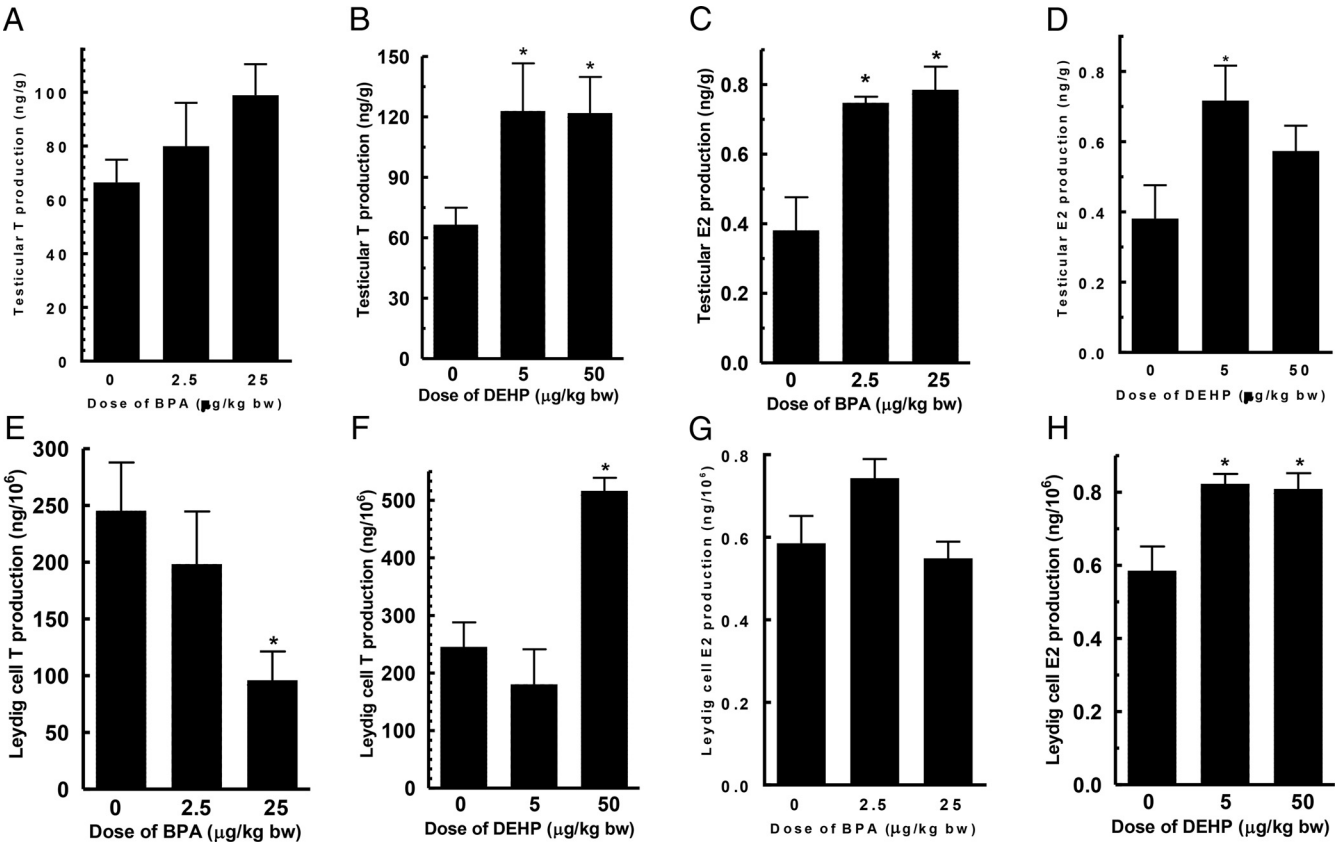

Figure 5. Male rats were exposed to BPA and DEHP by maternal gavage from GD 12 to GD 21. Testicular explants (A–D, n = 6 animals/group) and primary Leydig cell cultures (pooled from five to six animals per group; E–H) were obtained from adult male rats at 90 days of age. Tissues were incubated in DMEM/F-12 culture media for 3 hours to measure T and E2 concentrations in spent media by a RIA. *, $P < .05$ vs control.

by the smaller DEHP dose (Figure 5, C and D). Leydig cell T production was unaffected in adult male rats exposed to the smaller BPA and DEHP doses, but exposure to the greater BPA dose decreased ($P < .05$) Leydig cell T production, and the greater DEHP dose caused the opposite effect (Figure 5, E and F). On the other hand, Leydig cell E2 production was not affected by exposure to BPA but was increased by both DEHP doses compared with the control ($P < .05$) (Figure 5, G and H).

Prenatal exposure to BPA and DEHP affected global DNA methylation patterns associated with altered Dnmt expression

We performed assays to determine whether chemical exposures occurring during development-induced epigenetic changes by the assessment of global DNA methylation patterns in the adult testis. An analysis of genomic DNA showed that prenatal exposures to BPA and DEHP caused a decrease ($P < .05$) in 5hmc, but not 5mc, levels in testis of adult male rats at 90 days of age compared with the control (Figure 6, A, B, E, and F). Although testicular Dnmt3a protein expression was unchanged ($P > .05$) (Figure 6, C and G), Dnmt3b protein was greater ($P < .05$) in the testis of the adult male rats exposed to the greater BPA and DEHP doses than in the control group (Figure 6, D and H).

Discussion

Developmental exposures to BPA and DEHP affected gene expression in the developing and adult testes and altered gonadotropin subunit expression in the pituitary glands from prepubertal and adult male rats. The observed chemical exposure effects were associated with changes in serum steroid hormone concentrations and testicular androgen and estrogen secretion in the adult. The data also showed that prenatal exposures to BPA and DEHP affected global DNA methylation patterns and Dnmt protein expression in the testis of adult male rats.

Specifically, prenatal exposures to BPA and DEHP affected SF1, GATA-4, Sox9, and AMH protein expression in testis of infantile rats, although there were differences in BPA and DEHP effects. For example, the smaller, but not the greater, BPA dose caused a decrease, whereas DEHP had no effect on SF1 protein expression. On the other hand, the greater BPA dose caused an increase but DEHP caused the opposite effect on GATA-4 protein expression.

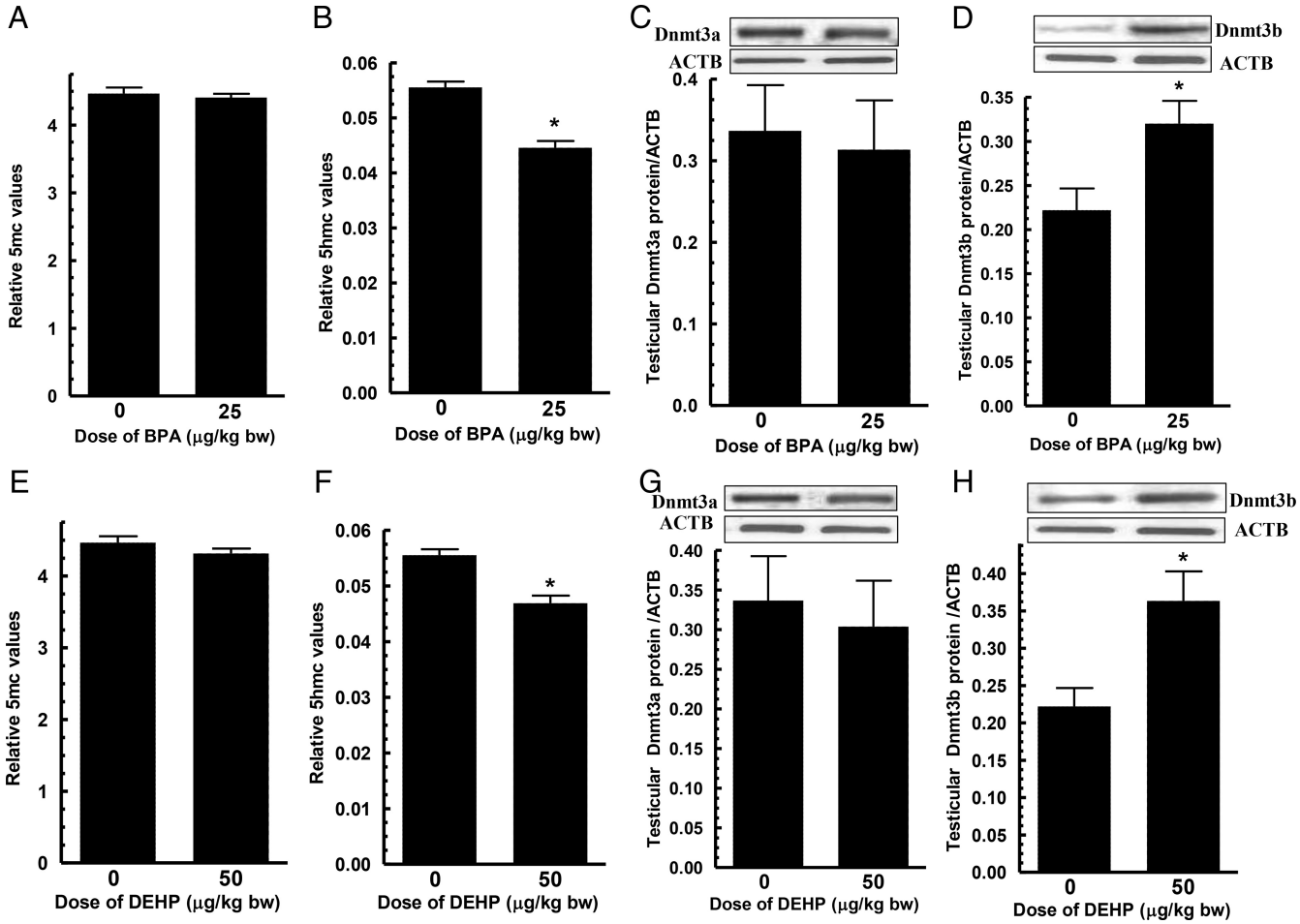

Figure 6. Male rats were exposed to BPA and DEHP by maternal gavage from GD 12 to GD 21. The testes from adult male rats at 90 days of age (n = 3) were processed to obtain genomic DNA and measure 5mc and 5hmc concentrations by liquid chromatography electrospray ionization tandem mass spectrometry with single-reaction monitoring (panels A, B, E, and F). Testicular tissue (n = 6) was analyzed for Dnmt3a and Dnmt3b protein expression using Western blotting procedures and antisera specific to rat Dnmt3a and Dnmt3b (panels C, D, G, and H). *, $P < .05$ vs control.

Interestingly, both test chemicals exerted similar effects on Sox9 and AMH expression. In this regard, Sox9-induced AMH overexpression has been described in patients suffering from the Sertoli cell-only syndrome (34–36). Thus, the presence of greater AMH protein levels in the serum and testis of BPA- and DEHP-exposed adult rats is possibly due to a failure of Sertoli cell maturation (36). This interpretation is supported by recent reports indicating that Sertoli cell-secreted AMH is a target for endocrine disruptors with estrogenic activity (35). BPA- and DEHP-induced increases in aromatase protein and E2 biosynthesis imply that both chemicals likely regulate the expression of the *Amh* gene, which possesses estrogen response elements in its promoter region (37). Although BPA and DEHP both caused an increase in the AR protein, signal transduction by the AR was not evaluated in the present study. Nevertheless, this is interesting because previous studies demonstrated that exposures to BPA and DEHP inhibited AR-mediated signaling (27, 36) and caused androgen insensitivity (38). Together, these observations imply that BPA and DEHP may cause their affects by modulation of both androgen- and estrogen-mediated activity.

An expression analysis of gonadotropin subunit protein in pituitary glands showed that prenatal exposures to BPA and DEHP increased LHβ and FSHβ protein in prepubertal male rats, but levels were decreased in the sexually mature animals. The negative feedback effects of sex steroids are mediated by interactions between AR and ESR response elements and other transcription factors that regulate FSHβ and LHβ expression (39). Previous studies indicated that serum AMH concentrations were decreased in prepubertal FSH-deficient mice (40), whereas AMH transcriptionally activated FSHβ and LHβ gene promoters in gonadotrope LβT2 cells and in adult rat pituitary glands expressing AMH receptor 2 receptors (41). In this regard, we found that serum AMH protein was increased in association with decreased pituitary LHβ and FSHβ protein in adult rats. Therefore, the impairment of Sertoli

cell development, reflected in part by increased AMH protein, is likely related to the chemical-induced disruption of gonadotropin action in the testis. The relationship between AMH and pituitary gonadotropins warrants further investigation, but it is possible that AMH is a target for toxicant-induced activity.

The impact of chemical exposure effects on Leydig cell androgen secretion on serum hormone concentrations is confounded by the capacity of chemical compounds to stimulate proliferative activity and affect Leydig cell numbers (18, 42). In this manner, changes in Leydig cell T secretion may not reflect in serum concentrations as seen in the present study. Similarly, testicular E2 was increased by BPA and DEHP, but Leydig cell E2 production was increased only in DEHP-exposed animals. We interpret these findings to mean that elevated serum E2 concentrations in the DEHP-exposed male rats was due to increased E2 secretion by Leydig cells, whereas increased testicular E2 secretion in the BPA-exposed group was due in part to contribution from other testicular cells, eg, Sertoli and germ cells (21). The increase in E2 production was associated with the induction of testicular aromatase expression by both chemicals, suggesting that BPA and DEHP altered E2 metabolism. Although the development of steroidogenic capacity in the testis was affected by exposure to BPA and DEHP, the expression of the Insl3 protein, a marker for Leydig cell differentiation, was unchanged when measured in 35-day-old male rats. However, BPA treatment increased Insl3 mRNA expression in the human but not rodent fetal testis (43), whereas BPA concentrations were negatively correlated with cord blood Insl3 concentrations in boys (44). The fetal testis was not analyzed for Insl3 expression in the present study, but there is evidence showing that EDCs may regulate factors other than Insl3 that affect development of steroid hormone secretion capacity. For example, AMH, which was affected by BPA and DEHP in the present study, has the capacity to regulate steroidogenic enzyme capacity in the testis (45, 46).

Developmental exposures to EDCs may affect DNA methylation patterns that later manifest as reproductive anomalies (47). For example, exposure of male rats to the antiandrogenic chemical vinclozolin induced epigenetic changes and caused testicular effects that were apparent in subsequent generations (48). We assessed global changes in DNA methylation in the testis of adult male rats using a selected reaction monitoring-based mass spectrometry assay (49). BPA and DEHP caused a decrease in 5hmc concentrations in association with altered expression of the Dnmt3b enzyme protein. Because DNA methylation is involved in gene regulation during development (50) and Dnmt3a and Dnmt3b are known to be catalytically active in vivo (51, 52), our findings suggest a dynamic regulation of DNA methylation and hydroxymethylation by low-dose exposures to BPA and DEHP that may impact gene regulation during reproductive development. However, genome-wide DNA sequence analysis using separated testicular cells to specify BPA and DEHP effects and identify areas of the genome associated with hypo- and/or hypermethylation events are warranted to extend the present findings.

In conclusion, the present data suggest that chemical exposures oc-

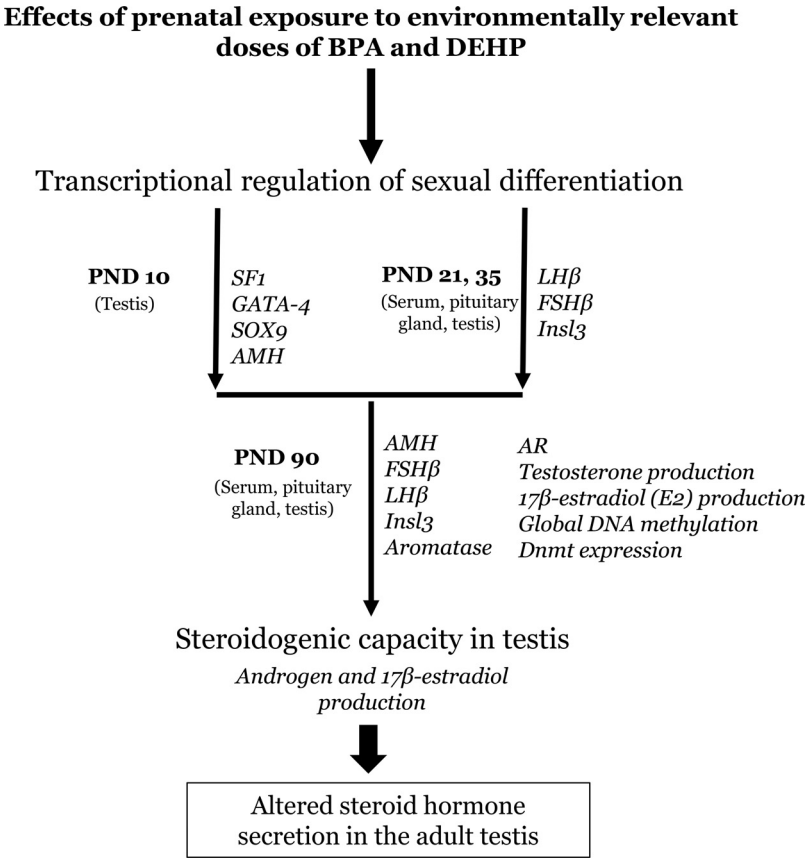

Figure 7. Chemical exposure effects on reproductive development were assessed by gene expression analysis, steroid hormone secretion, and global methylation patterns in serum, pituitary glands, and/or testis of male rats exposed to BPA and DEHP in the prenatal period. Altered steroid hormone secretion capacity in the adult testes occasioned by exposure to chemical agents possibly result from the disruption of the sexual differentiation process. Abbreviations are as defined in *Materials and Methods*.

curring in the fetal period are related to altered sexual differentiation events that impact gonadotropin subunit expression in pituitary glands and AMH and steroid hormone secretion in the testis (Figure 7). Furthermore, results indicated that low-dose chemical exposures that occur during development may affect global DNA methylation patterns (53). Together the data provide information relevant to the molecular mechanisms by which environmental chemicals disrupt male sexual differentiation. Data also implied that changes in steroid hormone secretion in the adult testes associated with varying exposure paradigms (21–23, 26, 54) are possibly related to chemical-induced effects exerted during development.

Acknowledgments

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References

1. **Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, et al.** Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocr Rev*. 2009;30(4):293–342.
2. **Krishnan AV, Stathis P, Permuth SF, Tokes L, Feldman D.** Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology*. 1993;132(6):2279–2286.
3. **Schonfelder G, Wittfoht W, Hopp H, Talsness CE, Paul M, Chahoud I.** Parent bisphenol A accumulation in the human maternal-fetal-placental unit. *Environ Health Perspect*. 2002;110(11):A703–A707.
4. **Calafat AM, Ye X, Wong LY, Reidy JA, Needham LL.** Exposure of the US population to bisphenol A and 4-tertiary-octylphenol: 2003–2004. *Environ Health Perspect*. 2008;116(1):39–44.
5. **Mylchreest E, Wallace DG, Cattley RC, Foster PM.** Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to Di(n-butyl) phthalate during late gestation. *Toxicol Sci*. 2000;55(1):143–151.
6. **Doull J, Cattley R, Elcombe C, et al.** A cancer risk assessment of di(2-ethylhexyl)phthalate: application of the new US EPA risk assessment guidelines. *Regul Toxicol Pharmacol*. 1999;29(3):327–357.
7. **Sharpe RM.** Pathways of endocrine disruption during male sexual differentiation and masculinization. *Best Pract Res Clin Endocrinol Metab*. 2006;20(1):91–110.
8. **Akingbemi BT.** Estrogen regulation of testicular function. *Reprod Biol Endocrinol*. 2005;3:51.
9. **Brucker-Davis F, Ducot B, Wagner-Mahler K, et al.** [Environmental pollutants in maternal milk and cryptorchidism]. *Gynecol Obstet Fertil*. 2008;36(9):840–847.
10. **Main KM, Mortensen GK, Kaleva MM, et al.** Human breast milk contamination with phthalates and alterations of endogenous reproductive hormones in infants three months of age. *Environ Health Perspect*. 2006;114(2):270–276.
11. **Swan SH, Main KM, Liu F, et al.** Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environ Health Perspect*. 2005;113(8):1056–1061.
12. **Lee MM, Donahoe PK.** Mullerian inhibiting substance: a gonadal hormone with multiple functions. *Endocr Rev*. 1993;14(2):152–164.
13. **Grinspon RP, Rey RA.** Anti-mullerian hormone and Sertoli cell function in paediatric male hypogonadism. *Horm Res Paediatr*. 2010;73(2):81–92.
14. **Cazorla O, Seck M, Pisselet C, et al.** Anti-Mullerian hormone (AMH) secretion in prepubertal and adult rams. *J Reprod Fertil*. 1998;112(2):259–266.
15. **Kucinskas L, Just W.** Human male sex determination and sexual differentiation: pathways, molecular interactions and genetic disorders. *Medicina (Kaunas)*. 2005;41(8):633–640.
16. **Harley VR, Goodfellow PN.** The biochemical role of SRY in sex determination. *Mol Reprod Dev*. 1994;39(2):184–193.
17. **de Santa Barbara P, Moniot B, Poulat F, Berta P.** Expression and subcellular localization of SF-1, SOX9, WT1, and AMH proteins during early human testicular development. *Dev Dyn*. 2000;217(3):293–298.
18. **Nanjappa MK, Simon L, Akingbemi BT.** The industrial chemical bisphenol A (BPA) interferes with proliferative activity and development of steroidogenic capacity in rat Leydig cells. *Biol Reprod*. 2012;86(5):135, 1–12.
19. **Zimmermann S, Steding G, Emmen JM, et al.** Targeted disruption of the Insl3 gene causes bilateral cryptorchidism. *Mol Endocrinol*. 1999;13(5):681–691.
20. **Brokken LJ, Adamsson A, Paranko J, Toppari J.** Antiandrogen exposure in utero disrupts expression of desert hedgehog and insulin-like factor 3 in the developing fetal rat testis. *Endocrinology*. 2009;150(1):445–451.
21. **Akingbemi BT, Sottas CM, Koulova AI, Klinefelter GR, Hardy MP.** Inhibition of testicular steroidogenesis by the xenoestrogen bisphenol A is associated with reduced pituitary luteinizing hormone secretion and decreased steroidogenic enzyme gene expression in rat Leydig cells. *Endocrinology*. 2004;145(2):592–603.
22. **Akingbemi BT, Ge R, Klinefelter GR, Zirkin BR, Hardy MP.** Phthalate-induced Leydig cell hyperplasia is associated with multiple endocrine disturbances. *Proc Natl Acad Sci USA*. 2004;101(3):775–780.
23. **Salian S, Doshi T, Vanage G.** Neonatal exposure of male rats to Bisphenol A impairs fertility and expression of Sertoli cell junctional proteins in the testis. *Toxicology*. 2009;265(1–2):56–67.
24. **Fisher JS, Macpherson S, Marchetti N, Sharpe RM.** Human 'testicular dysgenesis syndrome': a possible model using in-utero exposure of the rat to dibutyl phthalate. *Hum Reprod*. 2003;18(7):1383–1394.
25. **Vandenberg LN, Maffini MV, Sonnenschein C, Rubin BS, Soto AM.** Bisphenol-A and the great divide: a review of controversies in the field of endocrine disruption. *Endocr Rev*. 2009;30(1):75–95.
26. **Wolf C Jr, Lambright C, Mann P, et al.** Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p′-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane

sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicol Ind Health*. 1999;15(1–2):94–118.
27. **Teng C, Goodwin B, Shockley K, et al.** Bisphenol A affects androgen receptor function via multiple mechanisms. *Chem Biol Interact*. 2013;203(3):556–564.
28. **Klinefelter GR, Laskey JW, Winnik WM, et al.** Novel molecular targets associated with testicular dysgenesis induced by gestational exposure to diethylhexyl phthalate in the rat: a role for estradiol. *Reproduction*. 2012;144(6):747–761.
29. **Howdeshell KL, Peterman PH, Judy BM, et al.** Bisphenol A is released from used polycarbonate animal cages into water at room temperature. *Environ Health Perspect*. 2003;111(9):1180–1187.
30. **Ema M, Amano H, Itami T, Kawasaki H.** Teratogenic evaluation of di-n-butyl phthalate in rats. *Toxicol Lett*. 1993;69(2):197–203.
31. **Vecsey CG, Wimmer ME, Havekes R, et al.** Daily acclimation handling does not affect hippocampal long-term potentiation or cause chronic sleep deprivation in mice. *Sleep*. 2013;36(4):601–607.
32. **O'Shaughnessy PJ, Baker PJ, Heikkila M, Vainio S, McMahon AP.** Localization of 17β-hydroxysteroid dehydrogenase/17-ketosteroid reductase isoform expression in the developing mouse testis—androstenedione is the major androgen secreted by fetal/neonatal Leydig cells. *Endocrinology*. 2000;141(7):2631–2637.
33. **Payne AH, Downing JR, Wong KL.** Luteinizing hormone receptors and testosterone synthesis in two distinct populations of Leydig cells. *Endocrinology*. 1980;106(5):1424–1429.
34. **Cotinot C, Pailhoux E, Jaubert F, Fellous M.** Molecular genetics of sex determination. *Semin Reprod Med*. 2002;20(3):157–168.
35. **Chen G, Shinka T, Kinoshita K, Yan HT, Iwamoto T, Nakahori Y.** Roles of estrogen receptor α (ER α) in the regulation of the human Mullerian inhibitory substance (MIS) promoter. *J Med Invest*. 2003; 50(3–4):192–198.
36. **Lan KC, Chen YT, Chang C, et al.** Up-regulation of SOX9 in Sertoli cells from testiculopathic patients accounts for increasing anti-Mullerian hormone expression via impaired androgen receptor signaling. *PloS One*. 2013;8(10):e76303.
37. **Nagai A, Ikeda Y, Aso T, Eto K, Ikeda MA.** Exposure of neonatal rats to diethylstilbestrol affects the expression of genes involved in ovarian differentiation. *J Med Dent Sci*. 2003;50(1):35–40.
38. **Boukari K, Meduri G, Brailly-Tabard S, et al.** Lack of androgen receptor expression in Sertoli cells accounts for the absence of anti-Mullerian hormone repression during early human testis development. *J Clin Endocrinol Metab*. 2009;94(5):1818–1825.
39. **Lindzey J, Wetsel WC, Couse JF, Stoker T, Cooper R, Korach KS.** Effects of castration and chronic steroid treatments on hypothalamic gonadotropin-releasing hormone content and pituitary gonadotropins in male wild-type and estrogen receptor-α knockout mice. *Endocrinology*. 1998;139(10):4092–4101.
40. **Lukas-Croisier C, Lasala C, Nicaud J, et al.** Follicle-stimulating hormone increases testicular anti-Mullerian hormone (AMH) production through Sertoli cell proliferation and a nonclassical cyclic adenosine 5′-monophosphate-mediated activation of the AMH gene. *Mol Endocrinol*. 2003;17(4):550–561.
41. **Bedecarrats GY, O'Neill FH, Norwitz ER, Kaiser UB, Teixeira J.** Regulation of gonadotropin gene expression by Mullerian inhibiting substance. *Proc Natl Acad Sci USA*. 2003;100(16):9348–9353.
42. **Akingbemi BT, Youker RT, Sottas CM, et al.** Modulation of rat Leydig cell steroidogenic function by di(2-ethylhexyl)phthalate. *Biol Reprod*. 2001;65(4):1252–1259.
43. **N'Tumba-Byn T, Moison D, Lacroix M, et al.** Differential effects of bisphenol A and diethylstilbestrol on human, rat and mouse fetal Leydig cell function. *PloS One*. 2012;7(12):e51579.
44. **Chevalier N, Brucker-Davis F, Lahlou N, et al.** A negative correlation between insulin-like peptide 3 and bisphenol A in human cord blood suggests an effect of endocrine disruptors on testicular descent during fetal development. *Hum Reprod*. 2015;30(2):447–453.
45. **Behringer RR.** The in vivo roles of mullerian-inhibiting substance. *Curr Topics Dev Biol*. 1994;29:171–187.
46. **Behringer RR.** The mullerian inhibitor and mammalian sexual development. *Philos Trans R Soc London Series B Biol Sci*. 1995; 350(1333):285–288; discussion 289.
47. **Veiga-Lopez A, Luense LJ, Christenson LK, Padmanabhan V.** Developmental programming: gestational bisphenol-A treatment alters trajectory of fetal ovarian gene expression. *Endocrinology*. 2013;154(5):1873–1884.
48. **Anway MD, Cupp AS, Uzumcu M, Skinner MK.** Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science*. 2005;308(5727):1466–1469.
49. **Le T, Kim KP, Fan G, Faull KF.** A sensitive mass spectrometry method for simultaneous quantification of DNA methylation and hydroxymethylation levels in biological samples. *Anal Biochem*. 2011;412(2):203–209.
50. **Jue K, Benoit G, Alcivar-Warren AA, Trasler JM.** Developmental and hormonal regulation of DNA methyltransferase in the rat testis. *Biol Reprod*. 1995;52(6):1364–1371.
51. **La Salle S, Mertineit C, Taketo T, Moens PB, Bestor TH, Trasler JM.** Windows for sex-specific methylation marked by DNA methyltransferase expression profiles in mouse germ cells. *Dev Biol*. 2004;268(2):403–415.
52. **Singh S, Li SS.** Epigenetic effects of environmental chemicals bisphenol A and phthalates. *Int J Mol Sci*. 2012;13(8):10143–10153.
53. **Doerksen T, Benoit G, Trasler JM.** Deoxyribonucleic acid hypomethylation of male germ cells by mitotic and meiotic exposure to 5-azacytidine is associated with altered testicular histology. *Endocrinology*. 2000;141(9):3235–3244.
54. **Zhang L, Dong L, Ding S, et al.** Effects of n-butylparaben on steroidogenesis and spermatogenesis through changed E(2) levels in male rat offspring. *Environ Toxicol Pharmacol*. 2014;37(2):705–717.