ARTICLE
Journal of Cellular Biochemistry 116:1466–1477 (2015)

Journal of Cellular Biochemistry

# In Utero Exposure to Phthalate Downregulates Critical Genes in Leydig Cells of $F_1$ Male Progeny

Suganya Sekaran and Arunakaran Jagadeesan*

*Department of Endocrinology, Dr. ALM Post-Graduate Institute of Basic Medical Sciences, University of Madras, Chennai 600113, India*

## ABSTRACT

Phthalates are the largest group of environmental pollutants and are considered toxicant to the endocrine system. The present study was aimed to test the effect of in utero exposure of di(2-ethylhexyl)phthalate (DEHP) on Leydig cell steroidogenesis in $F_1$ male offspring's. Pregnant dams were oral gavaged with different doses (1, 10, and 100 mg/kg/day) of DEHP or olive oil during gestational Day 9–21. Serum testosterone (T) and estradiol ($E_2$) levels were significantly reduced in male offspring at 60 days of age. Our results also demonstrate a coordinate, dose-dependent disruption of genes involved in steroidogenesis. The gene expression of StAR, Cyp11a1, 3β-HSD, 17β-HSD, 5α-reductase and cytochrome P450 19a1 (or) aromatase (Cyp-19) were significantly decreased. The transcription factors like steroidogenic factor-1 (SF-1) and specific protein-1 (Sp-1) showed a significant decrease in 10 and 100 mg DEHP treatment group. DNA methylation analysis using bisulfite specific-methylation PCR shows hypermethylation in the SF-1 and Sp-1 promoter regions. Further to determine whether the DEHP-induced methylation changes were associated with increased DNA methyltransferase (Dnmt) levels, we measured the expression levels of Dnmt3a, Dnmt3b, Dnmt1, and Dnmt3l using real-time PCR and Western blot method. The mRNA and protein expressions of Dnmt3a, Dnmt3b, and Dnmt1 were stimulated in 10 and 100 mg DEHP treatment groups, whereas no significant change was seen in Dnmt3l expression, suggesting that increased Dnmt3a/b, Dnmt1 may cause DNA hypermethylation in testicular Leydig cells. Overall, these data suggest that gestational exposure to DEHP affects adult testicular function via altered methylation patterns. J. Cell. Biochem. 116: 1466–1477, 2015. 

**KEY WORDS:** DI(2-ETHYLHEXYL)PHTHALATE; ANDROGEN BIOSYNTHESIS; TRANSCRIPTION FACTORS; METHYLATION; DNA METHYLTRANSFERASE

Development of the normal male phenotype is dependent on androgen secreted by testes. Various endocrine disrupting compounds (EDCs), like dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), phthalates, bisphenol A (BPA), etc. are reported to disrupt androgen biosynthesis and actions [Skakkebaek et al., 2001]. Di(2-ethylhexyl)phthalate (DEHP) is a widely used plasticizer found at high concentrations in plastic products. DEHP is also found in medical devices [Calafat et al., 2004]. Frederiksen et al. [2007] reported that the main routes of DEHP exposure to humans are dermal contact and ingestion. Epidemiological studies provide evidence for the presence of DEHP in human samples such as umbilical cord blood [Latini et al., 2003], breast milk [Mortensen et al., 2005] and amniotic fluid [Huang et al., 2007]. Studies by Pan et al. [2006] reveal reduced concentrations of testosterone in workers at a polyvinyl chloride flooring factory. In addition phthalates have also been linked to reduced semen quality and fertility [Hauser et al., 2007; Pant et al., 2008]. An earlier report [Swan et al., 2005], shows high urinary concentrations of phthalate monoesters, the primary metabolites of phthalates and incidence of anomalies such as cryptorchidism and shortened anogenital distance (AGD) on newborn males suggesting that the Leydig cell function is disrupted.

Leydig cell androgen biosynthesis is a sequential process, which involves various enzymes to convert the substrate cholesterol into

Abbreviation used: AGD, anogenital distance; BS-MSPCR, bisulfite methylation-sensitive arbitrarily primed polymerase chain reaction; Cyp11a1, cytochrome P450 side chain cleavage enzyme; CYP17a1, 17α-hydroxylase/17, 20 lyase; Cyp-19, cytochrome P450 19a1 (or) aromatase; DEHP, di(2-ethylhexyl)phthalate; DHEA, dehydroepiandrosterone; DHT, 5α-dihydrotestosterone; DNMT, DNA methyltransferase; E2, estradiol; EDCs, endocrine disrupting compounds; MEHP, mono-2-ethylhexyl phthalate; HRP, horseradish peroxidase; PBR, peripheral-type benzodiazepine receptor; PND, postnatal day; RIPA, radio immuno precipitation assay buffer; SF-1, steroidogenic factor-1; Sp-1, specific protein-1; SR-B1, scavenger receptor class B type I; StAR, steroidogenic acute regulatory protein; T, testosterone; 3β-HSD, 3-beta-hydroxysteroid dehydrogenase; 17β-HSD, 17-beta-hydroxysteroid dehydrogenase.

Grant sponsor: UGC under SAP-DRS-III programme; Grant sponsor: UGC-BSR-RFSMS, New Delhi, India.

*Correspondence to: Dr. Arunakaran Jagadeesan, Department of Endocrinology, Dr. ALM Post-Graduate Institute of Basic Medical Sciences, University of Madras, Chennai 600113, India. E-mail: j_arunakaran@hotmail.com

Manuscript Received: 11 April 2014; Manuscript Accepted: 23 January 2015

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 3 February 2015

DOI 10.1002/jcb.25108 • © 2015 Wiley Periodicals, Inc.

testosterone through a series of steroidogenic steps [Payne and Hales, 2004]. The three initial enzymes responsible for conversion of cholesterol to testosterone include Cyp11a1, 3β-HSD, and Cyp17a1. Cyp11a1 cleaves the side chain of the C27 cholesterol to form C21 pregnenolone, which is converted to progesterone by 3β-HSD. From progesterone, Cyp17a1 forms 17-hydroxyprogesterone and the androstenedione. Scavenger receptor class B type I (SR-B1) mediates cholesterol uptake into the Leydig cells and the delivery of cholesterol molecules into the inner mitochondrial membrane is generally accepted to be the rate-determining step involving an interaction between the steroidogenic acute regulatory protein (StAR) and the peripheral-type benzodiazepine receptor (PBR) [Stocco, 2000]. In utero exposure of di-*n*-butyl phthalate (DBP) exhibited uniform decreases in the expression of steroidogenic genes, especially in a dose-dependent manner [Lehmann et al., 2004], which is consistent with decreased levels of testicular testosterone. Oral administration of DEHP to pregnant Sprague-Dawley rats at 750 mg/kg/day from gestation day (GD) 14 to postnatal day 3 (PND 3) reduced pup weight at birth, shortened male pup AGD, and reduced testis weights [Gray et al., 2000]. Borch et al. [2006] reported that, exposure of 100 and 300 mg/kg/day DEHP to pregnant Wistar rats from GD 7 to 21 produced male offspring that displayed reduced testicular testosterone levels as well as a reduction in the gene expression for StAR and Cyp11a1. The mechanism by which these genes are down-regulated by phthalate requires further study.

The recent findings [Bernal and Jirtle, 2010] reported that exposure to EDCs during the critical periods of prenatal and postnatal mammalian development, can interfere with hormonal signaling necessary for normal development through persistently altered gene expression. In addition, an earlier report [Rakyan and Whitelaw, 2003] provide an evidence that the ability of an external agent to induce a transgenerational effect requires stable chromosomal alterations or an epigenetic phenomenon such as DNA methylation. Herewith, it is clearly suggesting that there is a molecular link between environmental endocrine disruptor like DEHP and infertility. Studying the area of gene expression would be incomplete if epigenetic mechanisms of regulation are overlooked. The specific genes that have altered methylation states and are transmitted through the germ line remain to be fully elucidated. Study of such epigenomes may provide diagnostic and/or therapeutic markers to better understand specific diseases. Risk assessment for environmental toxicant exposure could consider the use of these genes as biomarkers.

The current study is hypothesized that gestational exposure of DEHP may disrupt testicular steroidogenesis through epigenetic alteration in Leydig cells of $F_1$ generation male rats. Objectives of the study is to evaluate the effect of DEHP on expression of genes involved in steroidogenic machinery, transcription regulation, and their methylation analysis using BS-MSPCR, and also to determine whether the DEHP-induced methylation changes were associated with increased Dnmt levels.

## MATERIALS AND METHODS

### CHEMICALS AND REAGENTS

Di-2-ethylhexyl phthalate or dioctyl phthalate ≥99.5% (D201154) CAS NO: 117-81-7, 500 ml, total RNA isolation reagent (TRIR), β-actin monoclonal antibody were purchased from Sigma–Aldrich Pvt Ltd. (St. Louis). One-step reverse transcriptase-polymerase chain reaction (RT-PCR) kit—Superscript-III Reverse Transcriptase was purchased Invitrogen and quantitative PCR (qPCR) reagents was purchased from Eurogentec (USA). The polyclonal StAR, Cyp11a1, 3β-HSD, 17β-HSD, 5α-reductase, Cyp-19, specific protein-1 (Sp-1), steroidogenic factor-1 (SF-1), and Dnmt3a/b, Dnmt1, Dnmt3l primary antibodies, and horseradish peroxidase (HRP)-conjugated anti-mouse and goat-anti-rabbit secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). PVDF membrane was purchased from Millipore (USA). EZ DNA Methylation™ kit (Catalog Nos. D5001) was purchased from Zymo Research (CA). All other chemicals were purchased from Amersham Biosciences Ltd. (UK) and Sisco Research Laboratories (Mumbai) of analytical grade (AR).

### ANIMALS

Animals were maintained as per the National Guidelines and Protocols approved by the Institutional Animal Ethical Committee (IAEC No: 01/01/10). Healthy adult female albino rats of Wistar strain *Rattus norvegicus*, weighing about 120 ± 20 g, were housed in polypropylene cages and maintained in an air-conditioned animal facility with constant 12 h/12 h dark and light cycle. They were fed with standard rat pelleted diet (Lipton India, Mumbai, India), and clean drinking water (reverse-osmosis) was made available ad libitum.

### TREATMENT

Male rats were exposed to DEHP by maternal gavage during gestation. Nulliparous rats were mated, and presence of sperm was taken as GD 0. Total no of dams used in this study = 72. Pregnant dams (n = 6–8/group) were gavaged once daily in the morning at 10:15 am regularly with either olive oil (control) or with 1, 10, and 100 mg/kg/day DEHP (Sigma–Aldrich, St. Louis, USA) dissolved in olive oil from GD 9 to GD 21 or until parturition [postnatal day (PND) 0]. The animals were weighed every 2 days, and the doses were adjusted accordingly. Male offspring were subsequently and randomly obtained from every dam in each group and analyzed at 60 days of age. Rats were sacrificed by decapitation and trunk blood was collected in tubes and centrifuged at 3000*g* for 10 min at 4°C and stored at −80°C for the estimation of serum hormones. Immediately, testes were also removed for the isolation of Leydig cells.

### ISOLATION, PURIFICATION, AND IDENTIFICATION OF LEYDIG CELLS

Leydig cells were isolated from testes of albino Wistar rats by enzymatic digestion and purified on a discontinuous Percoll gradient as described previously by Rigaudiere et al. [1988] with some modifications. The purified Leydig cells were identified by histochemical localization of 3β-HSD activity with dehydroepiandrosterone (DHEA) as the steroid substrate [Aldred and Cooke, 1983]. More than 95% were intensely stained.

### 3β-HSD ENZYME ACTIVITY

The activity of 3β-HSD in isolated Leydig cells was determined by the method described by Bergmeyer [1974]. In brief, the isolated Leydig cells were sonicated in ice-cold Tris–HCl buffer (pH 7.2) and

centrifuged at 16,000*g* for 5 min at 4°C. The supernatant was used as enzyme extract for the assay of 3β-HSD. The reaction mixture contained 0.6 ml pyrophosphate buffer (100 mM), 0.2 ml NAD (0.5 mM), 2 ml distilled water, and 0.1 ml dehydroisoandrosterone (0.1 mM). Immediately after the addition of enzyme extract, the absorbance was read at 340 nm in a spectrophotometer against blank for 5 min at 20-s intervals.

### ASSAY OF TESTOSTERONE, ESTRADIOL BY RADIOIMMUNOASSAY (RIA)

Serum T and $E_2$ were assayed by Immuchem™ Double antibody (liquid-phase) RIA kit obtained from ICN Biomedicals, Inc. (CA). For T, the intra-and inter-assay coefficients of variations were 4.6–9.1% and 7.5–11%, respectively. The cross-reactivity of the T antiserum with $E_2$ was <0.01%. T level was expressed as ng/ml. For $E_2$, the intra- and inter-assay coefficients of variations were 6.4–10.4% and 5.9–11.9%, respectively. The cross-reactivity with estriol was 1.51%. $E_2$ level was expressed as pg/ml.

### QUANTITATIVE REAL-TIME PCR (Q-PCR) ANALYSIS

The total RNA from isolated Leydig cells was extracted using Tri Reagent (Sigma) Chomczynski and Sacchi [1987]. Total RNA (2 μg) from each sample was reverse transcribed using a commercial Superscript III first strand cDNA synthesis kit (Invitrogen, USA) according to the manufacturer′s protocol. mRNA expression levels of StAR, Cyp11a1, 3β-HSD, 17β-HSD, 5α-reductase, Cyp-19, Sp-1, SF-1, and Dnmt′s were examined using real-time PCR. The list of primers and the internal control sequence are given in Table I. Real time-PCR was carried out in CFX Manager Bio-Rad PCR system (USA). Reaction was performed using MESA Green PCR master mix (It contains all the PCR components along with SYBR green dye.) Eurogentec. The specificity of the amplification product was determined by melting curve analysis for each primer pairs. The data were analyzed by comparative CT method and the fold change is calculated by $2^{-\Delta\Delta C_T}$ method described by [Schmittgen and Livak, 2008] using CFX Manager Version 2.1 (Bio-Rad).

### IMMUNOBLOT ANALYSIS

The isolated Leydig cells were lysed in radioimmunoprecipitation assay buffer (RIPA) buffer containing 1× protease inhibitor cocktail, and protein concentrations were determined by the method described by Lowry et al. [1951] using bovine serum albumin (BSA) as a standard. Cell lysate (50 μg) were mixed with 2× sample buffer and kept it in boiling water bath for 5 min. The sample mixture was run on 10 or 12% SDS–PAGE gel in 1× running gel buffer at 80 V and electro transferred to a PVDF membrane (Millipore) at 100 V for 1 h. The membranes were blocked in blocking buffer containing 5% albumin for 1 h. Then the blocked membranes were incubated with primary antibodies StAR, Cyp11a1, 3β-HSD, 17β-HSD, 5α-reductase, Cyp-19, Sp-1, SF-1, and Dnmt′s (Dnmt3a/b, Dnmt1, and Dnmt3l) (1:1,000) in Tris-buffered saline. After washing, the membranes were incubated with HRP conjugated anti-mouse IgG (1:5,000) and goat-anti rabbit IgG (1:5,000). Protein bands were detected using chemi luminescence system (ECL Kit) and quantified in Chemi Doc XRS Imaging System, Bio-Rad.

### METHYLATION-SPECIFIC PCR (MSP)

Briefly, genomic DNA was extracted from Leydig cells using the DNeasy kit (Qiagen, Valencia, CA). Genomic DNA (2μg) was bisulfate modified using EZ DNA Methylation™ kit (Zymo Research). The PCR

TABLE I. List of Primer Sequences Used in the Study

| Gene | Sense primer, anti-sense primer | Amplicon size (bp) | Gene bank accession number |
|---|---|---|---|
| Steroidogenic acute regulatory protein (STAR) | 5′-GCAGGCATGGCCACACAC-3′<br>5′-TGAGCAGCCAGCTGAGTT-3′ | 133 | NM_031558.3 |
| Cytochrome P450 side-chain cleavage enzyme (Cyp11A1) | 5′-CCAAGACTTTGGTGCAGG-3′<br>5′-AACACCCCAGCCAAAGCC-3′ | 146 | NM_017286.2 |
| 3-Beta-hydroxysteroid dehydrogenase (3β-HSD) | 5′-GTTGTCATCCACACCGCT-3′<br>5′-CTTCGACGCAGGCCTCCA-3′ | 112 | NM_001007719.3 |
| 17-Beta-hydroxysteroid dehydrogenase (HSD17b3) | 5′-ATGCTCCCCAACCTGCTCCCAA3′<br>5′-AGGCCTCTCCTTGATTCCA-3′ | 214 | NM_054007.1 |
| 5α-Reductase (Srd 5a1) | 5′-ACCAGAGCGAAGCAGCACCA-3′<br>5′-TGGGAGGCAACAGCGCTAACA-3′ | 116 | NM_017070.3 |
| Cytochrome P450 19a1 (or) aromatase (Cyp19a1) | 5′-AGGAGCCTTTACCTGCTCTTGGT-3′<br>5′-GCCCTTGAGTGGGTAGAGTGACG-3′ | 122 | NM_017085.2 |
| Specific protein-1 (Sp-1) | 5′-CATTCATTGCTGTCGGTC-3′<br>5′-CGGGGGCCACAGTTGCATT-3′ | 186 | NM_012655.2 |
| Steroidogenic factor-1 (SF-1) | 5′-GACAGCATCTTGCTGGTCAC-3′<br>5′-AATTTCACATCGAGGCTGAAGAG-3′ | 188 | NM_001191099.1 |
| DNA methyl transferease-1 (Dnmt-1) | 5′-CCAGATACCTACCGGTTATCG-3′<br>5′-TCCTTTAACTGCAGCTGAGGC-3′ | 201 | NM_053354.3 |
| DNA methyl transferease 3a (Dnmt-3a) | 5′-CTGAAATGGAAAGGGTGTTTGGC-3′<br>5′-CCATGTCCCTTACACACAAGC-3′ | 164 | NM_001003957.1 |
| DNA methyl transferease 3b (Dnmt-3b) | 5′-GTACTTCTGGGGTAACCTACC-3′<br>5′-GCAAACAGGTGTCTGATGACC-3′ | 333 | NM_001003959.1 |
| DNA methyl transferase 3L (Dnmt-3l) | 5′-AAGACCCATGAAACCTTGAACC-3′<br>5′-GTTGACTTCGTACCTGATGACCTC-3′ | 201 | NM_001003964.1 |
| 18s (internal control) | 5′-CGCTTCCTTACCTGGTTGAT-3′<br>5′-GAGCGACCAAAGGAACCATA-3′ | 134 | NR_046237.1 |

TABLE II. Primer List Used in Methylation-Sensitive PCR Experiments

| Gene symbol | Methylated/unmethylated | Sequence | $T_m$ (°C) | Amplicon size (bp) | CpGs/total Cs in primers |
|---|---|---|---|---|---|
| Sp-1 | MF | 5′-TTTTATCGTTTTAGAGAGAGAGCGA-3′ | 55 | 254 | 2/5 |
| | MR | 5′-GAACTCAAAAAAATCCTATCCGAA-3′ | 55 | 258 | 2/9 |
| | UF | 5′-TGGTTTTATAGTTTTAGAGAGAGAGTGA-3′ | 55 | | 2/5 |
| | UR | 5′-CAAACTCAAAAAAATCCTATCCAAA-3′ | 55 | | 2/9 |
| SF-1 | MF | 5′-AATTAGTGTTTCGTTAATCGGAGGTC-3′ | 55 | 266 | 3/6 |
| | MR | 5′-GAAAAACCCGTCCTCTCACG-3′ | 55 | 260 | 3/4 |
| | UF | 5′-TAGTGTTTTGTTAATTGGAGGTTGT-3′ | 55 | | 3/6 |
| | UR | 5′-CAAAAAACCCATCCTCTCACAC-3′ | 55 | | 3/4 |

mix consisted of master mix Fast Hot Start Ready mix (KAPA 2G$^{TM}$, USA) -10μl, forward primer 2μl, reverse primer 2μl and 4.5μl of bisulfate-treated DNA in 20μl total volume. MSP primer selection and primers listed in Table II were adopted from [Martinez-Arguelles et al., 2009]. The PCR conditions were: preincubation at 95°C (3 min), 40 cycles of 15 s at 95°C denaturation, 15 s annealing at 55°C, 1 min extension at 72°C. After PCR, 10 μl of PCR were mixed with loading dye and run on a 2% agarose gel containing ethidium bromide. Stained gels were visualized and digitalized using Bio-Rad Gel Doc Instrument (USA).

### STATISTICAL ANALYSIS

The data were subjected to statistical analysis using one-way analysis of variance (ANOVA) followed by Students Newman Keul's (SNK) test to assess the significance of individual variations between the control and treatment groups using a computer based software (SPSS 7.5). GraphPad Prism 5 was used for statistical analyses and graphics (GraphPad Software, Inc., La Jolla, CA), the significance was considered at level of $P < 0.05$.

## RESULTS

### GESTATIONAL EXPOSURE TO DEHP ON BODY WEIGHT AND RELATIVE ORGAN WEIGHT, ANOGENITAL DISTANCE (AGD) IN $F_1$ MALE WISTAR RATS (PND-60)

Figure 1A depicts the effect of DEHP on animal body weight. A dose-dependent decrease in body weight was observed in 1, 10, and 100 mg/kg b.wt/day DEHP treatment group. Figure 1B shows the effect of DEHP on testes and accessory sex organ weight. Dose-dependent decrease in relative organ weight was observed in testes. Epididymis shows decreased relative organ weight in 10 and 100 mg DEHP treatment group, whereas in ventral prostate and seminal vesicle only 100 mg DEHP treatment shows decrease in relative organ weight when compared to control. Male litters AGD were represented in Figure 1C at postnatal day 1, 7, 14, and 21 a significant decrease in AGD was observed in 10 and 100 mg/kg b.wt/day DEHP treatment group when compared to control.

### SERUM TESTOSTERONE (T), ESTRADIOL ($E_2$) LEVELS (PND-60)

The male primary steroid hormone, T is produced almost exclusively by Leydig cells in the testis. Studies by Hu et al. [2009] reveal, Leydig cell steroidogenic capacity serves as a hallmark for phthalate-mediated effects because these compounds, universally categorized as anti-androgens, affect T production. In the present study, we investigated the effect of gestational exposure to DEHP on serum T and $E_2$ levels in $F_1$ male Wistar rats at the age of 60 days (Fig. 1D,E). Our results showed dose-dependent decrease in the level of both T and $E_2$ with maximum decrease observed in 10 and 100 mg/kg b.wt/day DEHP-treated animals.

### EFFECT OF DEHP ON GENES INVOLVED IN STEROIDOGENIC MACHINERY

To find out the molecular mechanism behind the decreased serum T level we focused on expression level of genes involved in steroidogenic machinery. Figure 2A represents the effect of DEHP on StAR mRNA and protein expression in the Leydig cells. Ten and 100 mg/kg b.wt/day DEHP treated group showed a significant decrease in StAR expression when compared to control group. The first enzymatic step in steroidogenesis is the cholesterol being converted to pregnenolone by Cyp11a1, which take place in mitochondria [Payne and Hales, 2004]. Figure 2B represents the dose-dependent effect of DEHP on Cyp11a1 mRNA and protein expression in the Leydig cells. Ten milligram per kg b.wt/day DEHP treatment showed a significant decrease in Cyp11a1 expression and it further decreased in 100 mg/kg b.wt/day DEHP treated group when compared to control. The remaining steps take place in the smooth endoplasmic reticulum, where pregnenolone is converted to progesterone by 3β-HSD and then it is converted to androstenedione by P450c17, further metabolized to testosterone by 17β-HSD. Our results (Fig. 3A) registered a significant decrease in 3β-HSD mRNA and protein expression at 100 mg dose of DEHP treatment when compared to control. Whereas the activity of 3β-HSD showed a dose-dependent decrease when compared to control (Fig. 3B), 17β-HSD mRNA and protein expression were also reduced in 1, 10, and 100 mg/kg b.wt/day DEHP treated group when compared to control (Fig. 3C).

The more potent androgen 5α-dihydrotestosterone (DHT), obtained from circulating T by the enzyme 5α-reductase. The dose-dependent effect of DEHP in mRNA and protein expression of 5α-reductase (Fig. 4A) represents significantly decreased expression in 1, 10, and 100 mg/kg b.wt/day DEHP treated group when compared to control. To determine the variation in serum concentrations of $E_2$ the mRNA and protein expression of Cyp-19 was assessed, because $E_2$ is produced from T by the enzyme Cyp-19. The results (Fig. 4B) showed dose-dependent decrease of Cyp-19 in 1, 10, and 100 mg/kg b.wt/day DEHP-treated group when compared to control.

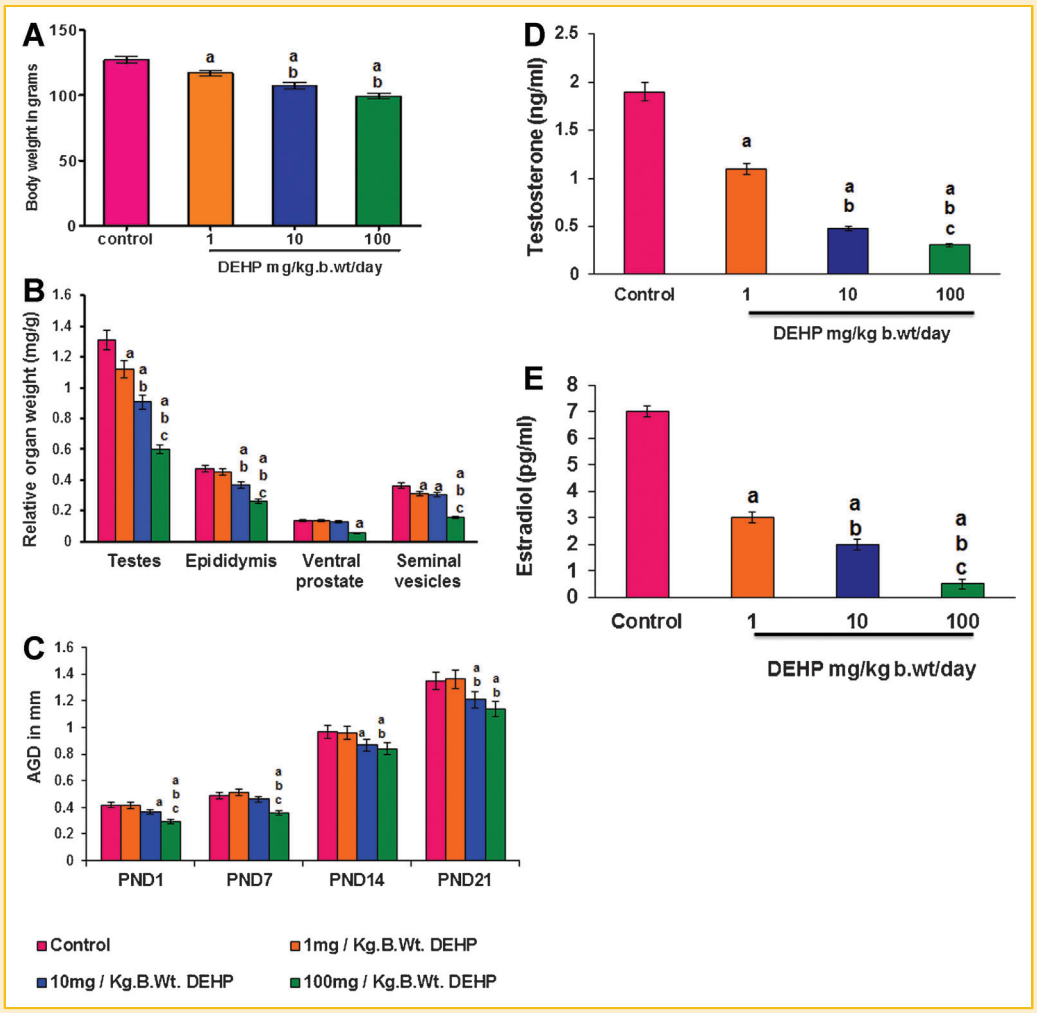


**Fig. 1.** Gestational exposure of di(2-ethylhexyl)phthalate (DEHP) on body weight at postnatal day (PND-60) indicated in (A), testis and accessory sex organ epididymis, ventral prostate, seminal vesicles relative weight at postnatal day (PND-60) indicated in (B). Measurement of anogenital distance (AGD) is considered to be a bioassay for androgen action. AGD at PND1, 7, 14, and 21 are indicated in (C). Each bar represents the mean ± SEM of three observations representing 16 animals. Serum testosterone (T) D: Estradiol ($E_2$). E: Levels in $F_1$ progeny male rats (PND-60). Serum T and $E_2$ were assayed by Immuchem™ Double antibody (liquid-phase) RIA kit. T level was expressed as ng/ml. $E_2$ level was expressed as pg/ml. Each bar represents mean ± SEM of six observations. Significance at $P < 0.05$, (a) compared with control; (b) compared with 1 mg DEHP; (c) compared with 10 mg DEHP.

### TRANSCRIPTIONAL REGULATION OF STEROIDOGENIC GENES

To show the importance of SF-1 and Sp-1 in the regulation of steroidogenic genes, we measured the expression levels of these transcription factors. Significant decreases in the mRNA expression of SF-1and Sp-1 (Fig. 5A) were observed in DEHP-treated group when compared to control. Whereas Western blot analysis showed a dose-dependent decrease in the protein expression of SF-1 and Sp-1 (Fig. 5B) in 1, 10, and 100 mg/kg b.wt/day DEHP-treated group.

### PROMOTER METHYLATION CHANGES IN TRANSCRIPTION FACTORS

In search of a mechanism to explain the decreased expression at PND-60 in response to DEHP exposure, we explored for epigenetic modifications of the transcription factors. MSP was used to screen for possible methylation changes in the transcription factor genes in the testicular Leydig cells. The results are shown in Figure 5C,D in which controls are compared with treated. Methylation increase was seen in the SF-1 (Fig. 5C) in response to 10, 100 mg/kg b.wt/day DEHP at PND 60. Sp-1 (Fig. 5D) methylation was also seen at PND-60 in response to 10, 100 mg/kg b.wt/day DEHP when compared to control and 1 mg/kg b.wt/day DEHP-treated group.

### DNMTs REGULATE DNA METHYLATION

Normal mammalian development requires the action of DNA methyltransferases (DNMTs) for the de novo establishment (DNMT3A and B) and maintenance (DNMT1) of DNA methylation within the genome. In addition, DNMT3L (DNMT3-like) that has similar sequence as DNMT3A and DNMT3B but lacking in enzymatic activity functions as a regulator of DNMT3A and DNMT3B [La Salle et al., 2004; Lucifero et al., 2004]. The expression level of these enzymes is highly regulated and peaks during specific stages of postnatal gonad

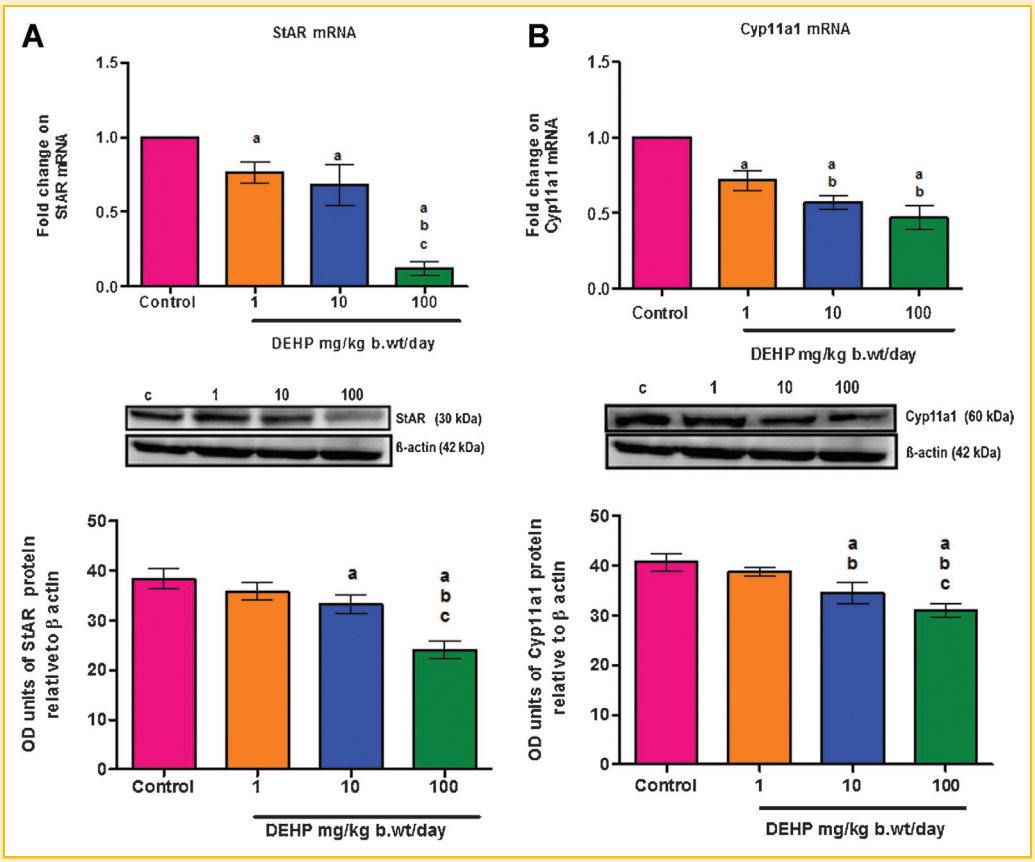


**Fig. 2.** Gestational exposure of di(2-ethylhexyl)phthalate (DEHP) on mRNA and protein expression of StAR (A). mRNA and protein expression of Cyp11a1 (B) in Leydig cells of $F_1$ progeny male rats. The mRNA expression was analyzed by isolating total RNA using total RNA isolation reagent (TRIR) and converting into complementary DNA (cDNA) using reverse transcriptase polymerase chain reaction, mRNA expression levels were examined using real-time PCR (Bio-Rad). The data were analyzed by comparative $C_T$ method and the fold change is calculated by $2^{-\Delta\Delta C_T}$ method. Total protein from whole cell lysates were subjected to Western blot analysis using antibodies directed against StAR and Cyp11a1. β-Actin served as an internal control. Each bar represents the mean ± SEM of three independent observations. Significance at $P < 0.05$, (a) compared with control; (b) compared with 1 mg DEHP; (c) compared with 10 mg DEHP.

development [Schaefer et al., 2007]. Therefore, measurement of the levels of Dnmt′s can be used to assess the epigenetic status of the testis. In our results Dnmt-1 expression was significantly increased in the 1, 10, and 100 mg/kg b.wt/day DEHP treatment doses when compared to control (Fig. 6A). Also the expression of Dnmt-3a/b was significantly increased in the same manner when compared to control (Fig. 6B). There were no significant alterations in the expression of Dnmt-3l in any DEHP treatment groups when compared to control (Fig. 6C).

## DISCUSSION

Our previous studies have demonstrated effects of DEHP in modulating the expression of Sertoli cell tight junctional and apoptotic protein through elevated free radical generation and oxidative stress [Sekaran et al., 2014]. The studies suggested that DEHP exposure can interfere with intercellular communication and possibly disrupt the process of spermatogenesis. These findings prompted the present investigation to examine possible effects of DEHP on Leydig cell functions, particularly for the synthesis and secretion of testosterone. In the first part of this study, the modulating effects of in utero exposure of DEHP on morphometric changes in $F_1$ male offspring were examined. We demonstrated in this study that DEHP interfered with the development of reproductive organs by observed decrease in animal body weight, and relative organ weight of testes, epididymis, ventral prostate, and seminal vesicle in agreement with previous report that in utero and lactational exposure to DEHP at 750 mg/kg caused dose-related reductions in body weight, the decrease was statistically significant from PND 63 through PND 105 and reduced the weight of prostate, an androgen-dependent gland, and sperm counts in the epididymis [Moore et al., 2001]. Andrade et al. [2006] reported that exposure of 475 mg/kg DEHP to adult rats showed significant decrease in serum T levels, and the weight of the seminal vesicle plus coagulating glands (androgen-dependent tissues) was also significantly reduced.

Measurement of AGD is considered to be a bioassay for fetal androgen action [Payne and Hales, 2004] and reduced AGD are considered to be a reliable marker of decreased T levels [Fisher, 2004]. In human new born boys, higher phthalate levels have been reported to be associated with decreased AGDs [Swan et al., 2005]. Studies by

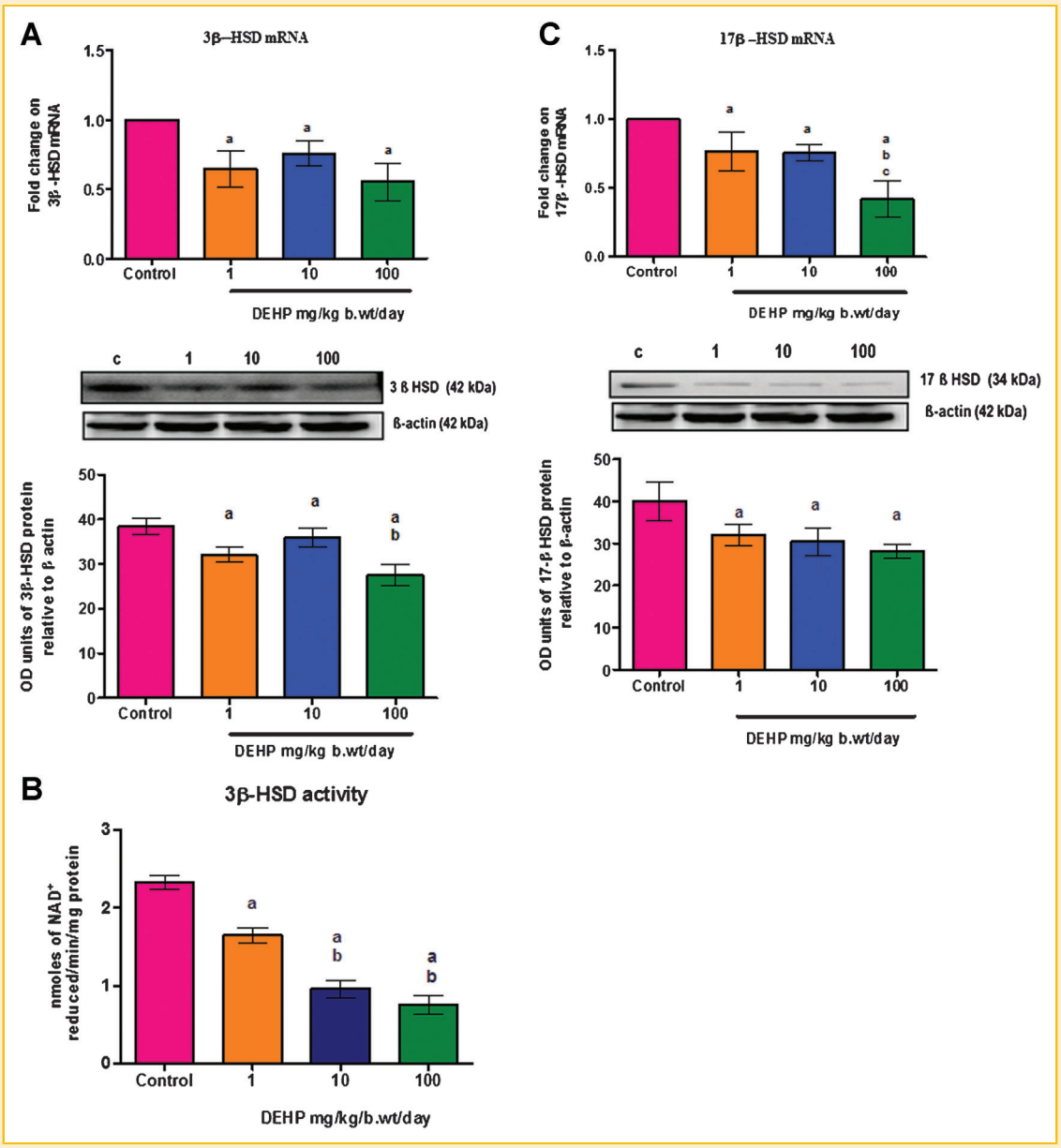


**Fig. 3.** Gestational exposure of di(2-ethylhexyl)phthalate (DEHP) on mRNA and protein expression of 3β-HSD (A). 3β-HSD activity (B), mRNA and protein expression of 17β-HSD (C) in Leydig cells of $F_1$ progeny male rats. 3β-HSD activity was assessed by spectrophotometric method with dehydroepiandrosterone (DHEA) as the steroid substrate. mRNA expression was analyzed by isolating total RNA using total RNA isolation reagent (TRIR) and converting into complementary DNA (cDNA) using reverse transcriptase polymerase chain reaction, mRNA expression levels were examined using real-time PCR (Bio-Rad). The data were analyzed by comparative $C_T$ method and the fold change is calculated by $2^{-\Delta\Delta C_T}$ method. Total protein from whole cell lysates were subjected to Western blot analysis using antibodies directed against 3β-HSD and 17β-HSD. β-Actin served as an internal control. Each bar represents the mean ± SEM of three independent observations. Significance at $P < 0.05$, (a) compared with control; (b) compared with 1 mg DEHP; (c) compared with 10 mg DEHP.

Clark et al. [1994] reveal exposure to high doses (405 and 500 mg/kg/day) of DEHP during lactation showed significant reduction of AGD in adult male offspring. In addition, a decline evoked by this phthalate in T synthesis result in decreased AGD value. In the current investigation in utero exposure to DEHP resulted in a significantly decreased AGD in $F_1$ male Wistar rats, which correlate with the observed decline in serum levels of T. Studies also reported that in utero exposures to DEHP (100 mg/kg) alone caused significant decreases of serum T levels postnatally [Akingbemi et al., 2000]. The decreased Leydig cell capacity was correlated with downregulation of most genes related to Leydig cell regulation and steroidogenic pathways (such as SR-B1, Star, Cyp17a1, and Hsd17b3) at PND 21. Previous studies does not show any correlation between reduced serum T levels and genes related to Leydig cell steroidogenesis in the adult testis after the cessation of DEHP exposures during in utero. In the present study we propose that, one of the possible causes may be

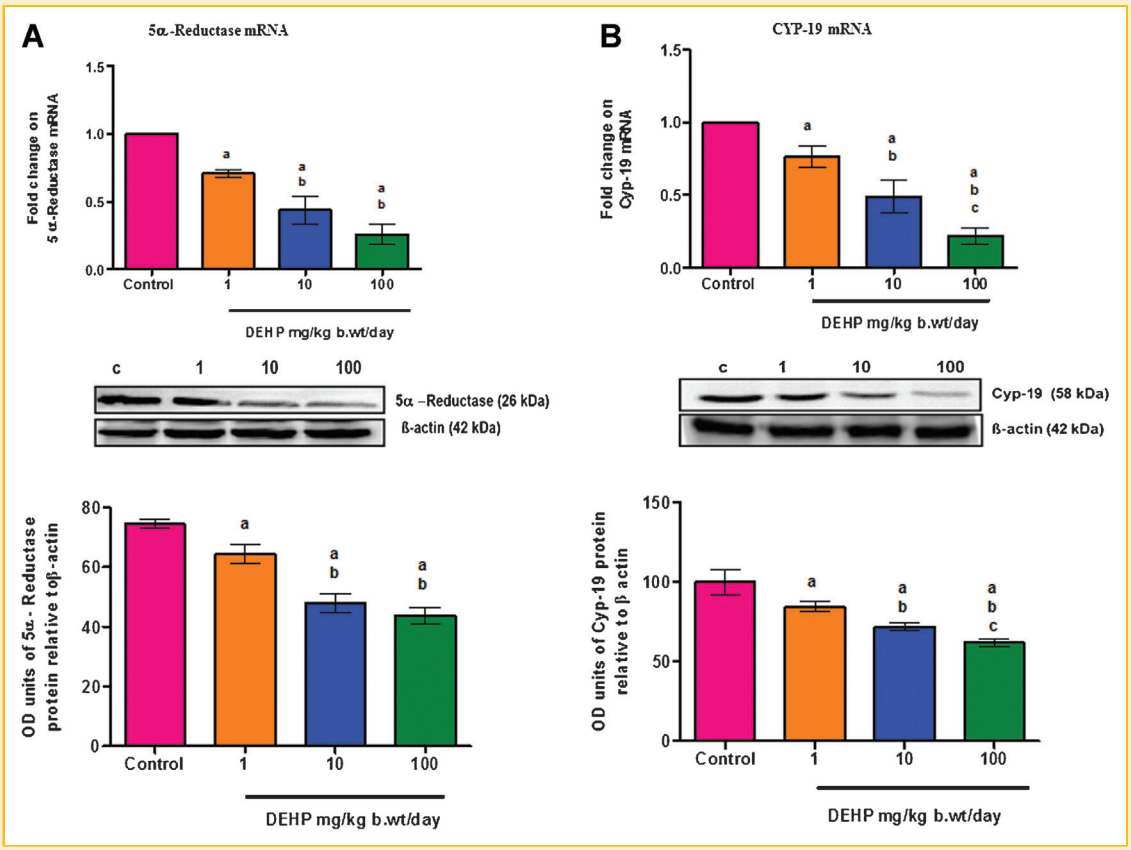


**Fig. 4.** Gestational exposure of di(2-ethylhexyl)phthalate (DEHP) on mRNA and protein expression of 5α-reductase (A). mRNA and protein expression of Cyp-19 (B) in Leydig cells of $F_1$ progeny male rats. mRNA expression was analyzed by isolating total RNA using total RNA isolation reagent (TRIR) and converting into complementary DNA (cDNA) using reverse transcriptase polymerase chain reaction, mRNA expression levels were examined using real-time PCR (Bio-Rad). The data were analyzed by comparative $C_T$ method and the fold change is calculated by $2^{-\Delta\Delta C_T}$ method. Total protein from whole cell lysates were subjected to Western blot analysis using antibodies directed against 5α-reductase and Cyp-19. β-Actin served as an internal control. Each bar represents the mean ± SEM of three independent observations. Each bar represents the mean ± SEM of three independent observations. Significance at $P < 0.05$, (a) compared with control; (b) compared with 1 mg DEHP; (c) compared with 10 mg DEHP.

defect at the expression level of genes related to Leydig cell steroidogenesis regulation.

In Leydig cell steroidogenesis, the substrate cholesterol is converted into T through a series of steroidogenic steps catalysed by different enzymes [Payne and Hales, 2004]. The delivery of free cholesterol to the inner mitochondrial membrane is required to initiate the steroidogenic process. This is the rate-limiting step of steroidogenesis and is mediated by the StAR protein [Clark et al., 1994]. The first enzymatic step in steroidogenesis is cholesterol being converted to pregnenolone by Cyp11a1 [Payne and Hales, 2004]. In the current study in utero exposure of DEHP down regulated the gene expression of StAR with a maximum decrease observed in 100 mg DEHP-treated rats. This is in agreement with inhibitory effect of MEHP primary metabolite of DEHP on steroidogenesis in immature and adult Leydig cell in vitro mediated by StAR protein [Vo et al., 2009]. Cyp11a1 a monooxygenase, is necessary for the synthesis of cholesterol and steroids, and a decrease in the expression level of Cyp11a1 following in utero DEHP exposure was identified in the present study, in line with the previous demonstrations [Borch et al., 2006]. Studies also stated that exposure to other EDCs like TCDD show decreased pregnenolone production in the testis of treated rats by inhibiting the mobilization of cholesterol by Cyp11a1 [Moore et al., 1991]. We also measured the expression of 3β-HSD and 17β-HSD, which showed a decreased mRNA and protein expression at PND-60 after exposure to 1, 10, and 100 mg/kg b.wt/day DEHP to Wistar rat on GD 9-21. In addition in the current investigation, we observed a reduced 3β-HSD activity. Exposure of rats to 200 mg/kg/day DEHP caused a 77% decrease in the activity of the steroidogenic enzyme 17β-HSD, and reduced Leydig cell T production to 50% of control [Akingbemi et al., 2001]. The decreased gene expression levels in DEHP exposed Leydig cells were also accompanied by reduced activities of steroidogenic enzymes suggesting the possible inhibitory effects of DEHP or its metabolite on steroidogenic enzymes gene expression.

Phthalate esters also alter the expression of testosterone and estrogen metabolism genes [Kim et al., 2003]. Our study showed decreased 5α-reductase expression which converts T into a more potent androgen DHT, which agrees with the previous study that exposure to MEHP alone, decreased 5α-reductase activity in immature Leydig cells [Svechnikov et al., 2008].

The expression level of Cyp19a1, which involves in the conversion of T to $E_2$ was decreased in 1, 10, and 100 mg/kg b wt/day DEHP

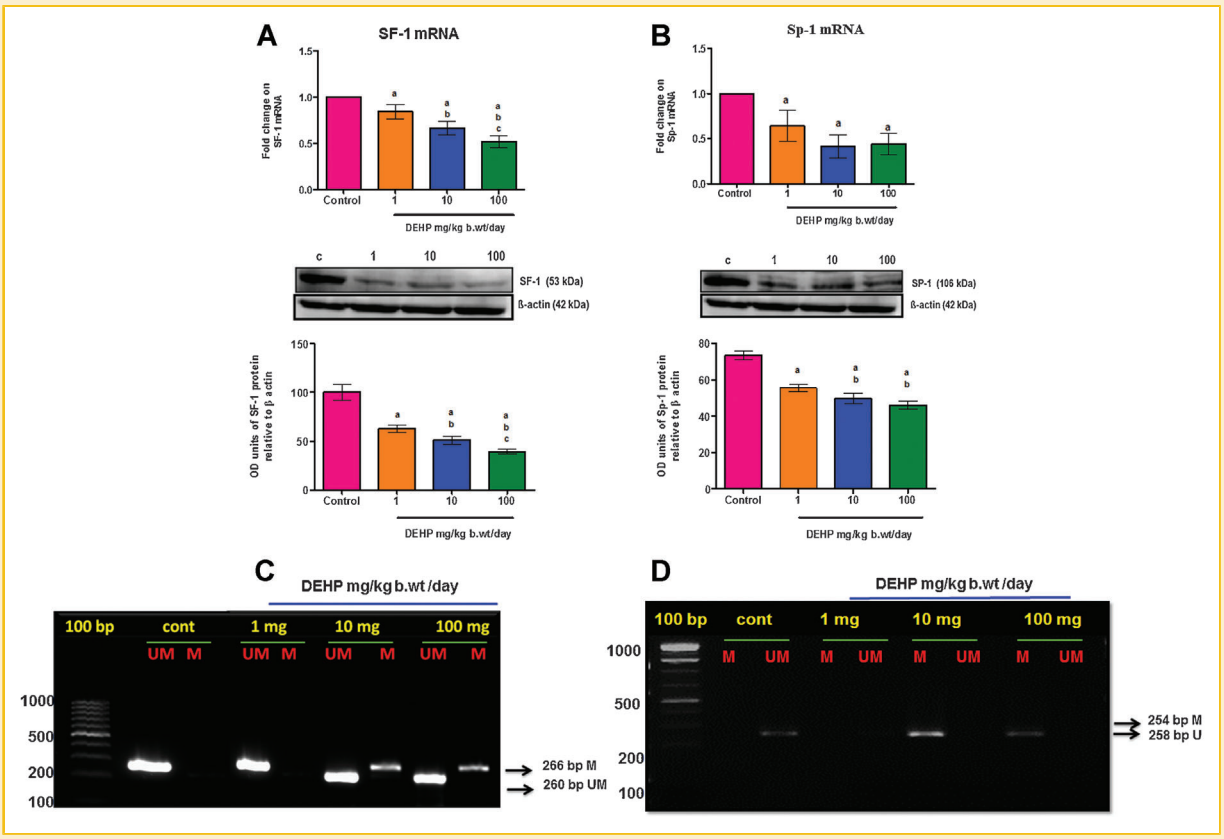


**Fig. 5.** Gestational exposure of di(2-ethylhexyl)phthalate (DEHP) on mRNA and protein expression of SF-1 (A). mRNA and protein expression of Sp-1 (B) in Leydig cells of $F_1$ progeny male rats. mRNA expression was analyzed by isolating total RNA using total RNA isolation reagent (TRIR) and converting into complementary DNA (cDNA) using reverse transcriptase polymerase chain reaction, mRNA expression levels were examined using real-time PCR (Bio-Rad). The data were analyzed by comparative $C_T$ method and the fold change is calculated by $2^{-\Delta\Delta C_T}$ method. Total protein from whole cell lysates were subjected to Western blot analysis using antibodies directed against SF-1 and Sp-1. β-Actin served as an internal control. Each bar represents the mean ± SEM of three independent observations. Significance at $P < 0.05$, (a) compared with control; (b) compared with 1 mg DEHP; (c) compared with 10 mg DEHP. Methylation changes in SF-1 promoter (C) and Sp-1 promoter (D) Leydig cells of $F_1$ progeny male rats. Briefly, genomic DNA was extracted from Leydig cells using the DNeasy kit. Two micrograms of genomic DNA was bisulfate modified using EZ DNA Methylation™ kit. Bisulfate-treated DNA was amplified using Fast Hot Start Ready mix (KAPA 2G™, USA) with specific methylated and unmethylated primers for SF-1 and Sp-1 promoter region. PCR products were mixed with loading dye and run on a 2% agarose gel containing ethidium bromide. Stained gels were visualized and digitalized using Bio-Rad Gel Doc Instrument. Results shown are from the offspring of three dams per treatment point, with each sample processed in triplicate. Lane 1:100 bp marker; lane 2: control; lane 3: 1 mg DEHP; lane 4: 10 mg DEHP; lane 5: 100 mg DEHP; UM, unmethylated; M, methylated.

exposure in a dose-dependent manner. Thus, in addition to the decreased serum T level, under expression of Cyp19a1 also contributed to the observed decrease in the level of serum $E_2$. DBP and DEHP have similar mode of action in development of the male reproductive tract by which they exert their effects on the regulation of gene expression involved in cholesterol transport and T synthesis. Studies have been reported that a significant decrease in the expression of Cyp19a1 gene, a member of the family of sex determination and steroid metabolism related genes, is observed when fetal testes are maternally exposed to DBP [Ryu et al., 2007]. Moreover, studies by Borch et al. [2006] reveal DEHP is thought to activate Peroxisome proliferator-activated receptor (PPAR), leading to downregulation of SR-B1 and PBR or SF-1 that consequently regulate steroidogenesis-related genes.

The nuclear receptor SF-1 plays a prominent role in the development and differentiation of steroidogenic tissues and controls the expression of steroidogenic enzymes and cholesterol transporters required for steroidogenesis [Val et al., 2003]. Lehmann et al. [2004] reported that in utero exposure of DBP exhibited decreased levels of testicular T, which is consistent with decreases in the expression of steroidogenic genes in a dose-dependent manner. Studies [Sugawara et al., 2000] have provided strong evidence of interaction between Sp1, a global transcription factor, and SF-1 in the regulation of human StAR gene transcription. Other genes altered by DBP exposure including SR-B1, P450scc, CYP17a1, and 3β-HSD, also require SF-1 for basal promoter activity and for cyclic AMP induction [Shultz et al., 2001]. In the present study, both the mRNA and protein expression of SF-1 and Sp-1 decreased in a dose-dependent manner at PND-60, which correlates with decreased expression of genes regulated by these transcription factors.

Earlier reports [Newbold, 2004; Ho et al., 2006; Crain et al., 2008; Soto et al., 2008] showed developmental exposure to EDCs, cause dysfunction of the organ systems like mammary gland, prostate, uterus, and ovary in the adult stage in associated with alterations in

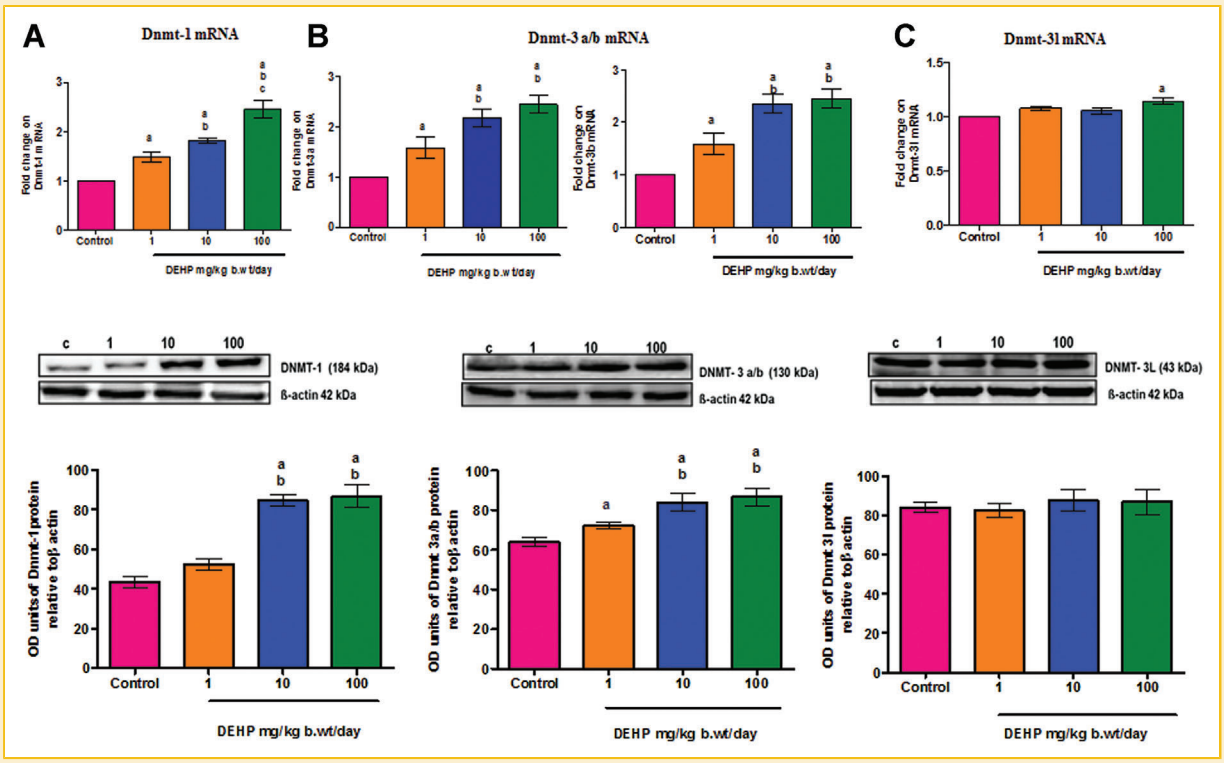


**Fig. 6.** Gestational exposure of di(2-ethylhexyl)phthalate (DEHP) on mRNA and protein expression of Dnmt-1(A), Dnmt-3a/b (B) and Dnmt-3L (C) in Leydig cells of $F_1$ progeny male rats. mRNA expression was analyzed by isolating total RNA using total RNA isolation reagent (TRIR) and converting into complementary DNA (cDNA) using reverse transcriptase polymerase chain reaction, mRNA expression levels were examined using real-time PCR (Bio-Rad). The data were analyzed by comparative $C_T$ method and the fold change is calculated by $2^{-\Delta\Delta C_T}$ method. Total protein from whole cell lysates were subjected to Western blot analysis using antibodies directed against Dnmt-1, Dnmt-3a/b, and Dnmt-3l. β-Actin served as an internal control. Each bar represents the mean ± SEM of three independent observations. Significance at $P < 0.05$, (a) compared with control; (b) compared with 1 mg DEHP; (c) compared with 10 mg DEHP.

DNA methylation patterns. It is likely that similar defects could occur in the testis [Anway et al., 2005]. Studies reported that in utero exposure to DEHP causes decreased expression of mineralocorticoid receptor (MR) targeted gene in the adult rat (PND-60), possibly through an epigenetic-mediated mechanism [Martinez-Arguelles et al., 2009].

We hypothesized that reduced T formation by the adult testis after in utero exposure to DEHP might be due to an epigenetic alteration of genes regulating T biosynthesis. We used MSP to screen methylation of promoter areas of SF-1 and Sp-1 gene. Interestingly, dose-dependent changes were observed in the expression pattern of methylated and unmethylated gene in 10 and 100 mg DEHP/kg b.wt/day when compared to control and 1 mg DEHP/kg b.wt/day. Even though our result showed altered expression of unmethylated and methylated genes in control and DEHP treatment group, this observation alone cannot be interpreted to mean that there are effects on gene expression.

Studies by Wu et al. [2010] reported that maternal exposure to DEHP in mice significantly increased DNA methylation levels through increased expression of DNMTs. That they reported that, changes in DNA methylation may play an important role in abnormal testicular function caused by environmental factors such as maternal exposure to DEHP, which may be one of the mechanisms of DEHP-mediated testicular toxicity. In the present study, the mRNA and protein expression of Dnmt1, Dnmt3a/b increased in a dose dependent manner when compared to control. Therefore, it could contribute to the altered methylation pattern of SF-1 and Sp-1 gene, which result in decreased expression of these genes and contribute to abnormal testicular function.

In conclusion, the present study shows that in utero exposure of DEHP caused adult Leydig cell dysfunction in a dose dependent manner through epigenetic-mediated mechanism.

## ACKNOWLEDGMENT

Financial assistance to the Department of Endocrinology from UGC under SAP-DRS-III programme, UGC-BSR-RFSMS, New Delhi, India is gratefully acknowledged.

## REFERENCES

Akingbemi BT, Ge RS, Klinefelter GR, Gunsalus GL, Hardy MP. 2000. A metabolite of methoxychlor, 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane, reduces testosterone biosynthesis in rat leydig cells through suppression of steady-state messenger ribonucleic acid levels of the cholesterol side-chain cleavage enzyme. Biol Reprod 62:571–578.

Akingbemi BT, Youker RT, Sottas CM, Ge R, Katz E, Klinefelter GR, Zirkin BR, Hardy MP. 2001. Modulation of rat Leydig cell steroidogenic function by di(2-ethylhexyl)phthalate. Biol Reprod 65:1252–1259.

Aldred LF, Cooke BA. 1983. The effect of cell damage on the density and steroidogenic capacity of rat testis Leydig cells, using an NADH exclusion test for determination of viability. J Steroid Biochem 18:411–414.

Andrade AJ, Grande SW, Talsness CE, Gericke C, Grote K, Golombiewski A, Sterner-Kock A, Chahoud I. 2006. A dose response study following in utero and lactational exposure to di-(2-ethylhexyl)phthalate (DEHP): Reproductive effects on adult male offspring rats. Toxicology 228:85–97.

Anway MD, Cupp AS, Uzumcu M, Skinner MK. 2005. Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 308: 1466–1469.

Bergmeyer M. 1974. Steroid dehydrogenase. In: Bergmeyer H, editor. Methods of enzymatic analysis. New York: Academic Press. pp 476–477.

Bernal AJ, Jirtle RL. 2010. Epigenomic disruption: The effects of early developmental exposures. Birth Defects Res A Clin Mol Teratol 88:938–944.

Borch J, Metzdorff SB, Vinggaard AM, Brokken L, Dalgaard M. 2006. Mechanisms underlying the anti-androgenic effects of diethylhexyl phthalate in fetal rat testis. Toxicology 223:144–155.

Calafat AM, Needham LL, Silva MJ, Lambert G. 2004. Exposure to di-(2-ethylhexyl)phthalate among premature neonates in a neonatal intensive care unit. Pediatrics 113:e429–e434.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochem 162:156–159.

Clark BJ, Wells J, King SR, Stocco DM. 1994. The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). J Biol Chem 269:28314–28322.

Crain DA, Janssen SJ, Edwards TM, Heindel J, Ho SM, Hunt P, Iguchi T, Juul A, McLachlan JA, Schwartz J, Skakkebaek N, Soto AM, Swan S, Walker C, Woodruff TK, Woodruff TJ, Giudice LC, Guillette LJ, Jr. 2008. Female reproductive disorders: The roles of endocrine-disrupting compounds and developmental timing. Fertil Steril 90:911–940.

Fisher JS. 2004. Environmental anti-androgens and male reproductive health: Focus on phthalates and testicular dysgenesis syndrome. Reproduction 127:305–315.

Frederiksen H, Skakkebaek NE, Andersson AM. 2007. Metabolism of phthalates in humans. Mol Nutr Food Res 51:899–911.

Gray LE, Jr, Ostby J, Furr J, Price M, Veeramachaneni DN, Parks L. 2000. Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. Toxicol Sci 58:350–365.

Hauser R, Meeker JD, Singh NP, Silva MJ, Ryan L, Duty S, Calafat AM. 2007. DNA damage in human sperm is related to urinary levels of phthalate monoester and oxidative metabolites. Hum Reprod 22:688–695.

Ho SM, Tang WY, Belmonte de Frausto J, Prins GS. 2006. Developmental exposure to estradiol and bisphenol A increases susceptibility to prostate carcinogenesis and epigenetically regulates phosphodiesterase type 4 variant 4. Cancer Res 66:5624–5632.

Hu GX, Lian QQ, Ge RS, Hardy DO, Li XK. 2009. Phthalate-induced testicular dysgenesis syndrome: Leydig cell influence. Trends Endocrinol Metab 20: 139–145.

Huang PC, Kuo PL, Guo YL, Liao PC, Lee CC. 2007. Associations between urinary phthalate monoesters and thyroid hormones in pregnant women. Hum Reprod 22:2715–2722.

Kim HS, Saito K, Ishizuka M, Kazusaka A, Fujita S. 2003. Short period exposure to di-(2-ethylhexyl)phthalate regulates testosterone metabolism in testis of prepubertal rats. Arch Toxicol 77:446–451.

La Salle S, Mertineit C, Taketo T, Moens PB, Bestor TH, Trasler JM. 2004. Windows for sex-specific methylation marked by DNA methyltransferase expression profiles in mouse germ cells. Dev Biol 268:403–415.

Latini G, De Felice C, Presta G, Del Vecchio A, Paris I, Ruggieri F, Mazzeo P. 2003. In utero exposure to di-(2-ethylhexyl)phthalate and duration of human pregnancy. Environ Health Perspect 111:1783–1785.

Lehmann KP, Phillips S, Sar M, Foster PM, Gaido KW. 2004. Dose-dependent alterations in gene expression and testosterone synthesis in the fetal testes of male rats exposed to di (n-butyl)phthalate. Toxicol Sci 81:60–68.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275.

Lucifero D, Chaillet JR, Trasler JM. 2004. Potential significance of genomic imprinting defects for reproduction and assisted reproductive technology. Hum Reprod Update 10:3–18.

Martinez-Arguelles DB, Culty M, Zirkin BR, Papadopoulos V. 2009. In utero exposure to di-(2-ethylhexyl)phthalate decreases mineralocorticoid receptor expression in the adult testis. Endocrinology 150:5575–5585.

Moore RW, Jefcoate CR, Peterson RE. 1991. 2,3,7,8-Tetrachlorodibenzo-p-dioxin inhibits steroidogenesis in the rat testis by inhibiting the mobilization of cholesterol to cytochrome P450scc. Toxicol Appl Pharmacol 109:85–97.

Moore RW, Rudy TA, Lin TM, Ko K, Peterson RE. 2001. Abnormalities of sexual development in male rats with in utero and lactational exposure to the antiandrogenic plasticizer Di(2-ethylhexyl)phthalate. Environ Health Perspect 109:229–237.

Mortensen GK, Main KM, Andersson AM, Leffers H, Skakkebaek NE. 2005. Determination of phthalate monoesters in human milk, consumer milk, and infant formula by tandem mass spectrometry (LC–MS–MS). Anal Bioanal Chem 382:1084–1092.

Newbold RR. 2004. Lessons learned from perinatal exposure to diethylstilbestrol. Toxicol Appl Pharmacol 199:142–150.

Pan G, Hanaoka T, Yoshimura M, Zhang S, Wang P, Tsukino H, Inoue K, Nakazawa H, Tsugane S, Takahashi K. 2006. Decreased serum free testosterone in workers exposed to high levels of di-n-butyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP): A cross-sectional study in China. Environ Health Perspect 114:1643–1648.

Pant N, Shukla M, Kumar Patel D, Shukla Y, Mathur N, Kumar Gupta Y, Saxena DK. 2008. Correlation of phthalate exposures with semen quality. Toxicol Appl Pharmacol 231:112–116.

Payne AH, Hales DB. 2004. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr Rev 25:947–970.

Rakyan V, Whitelaw E. 2003. Transgenerational epigenetic inheritance. Curr Biol 13:R6.

Rigaudiere N, Loubassou S, Grizard G, Boucher D. 1988. Characterization of insulin binding and comparative action of insulin and insulin-like growth factor I on purified Leydig cells from the adult rat. Int J Androl 11:165–178.

Ryu JY, Lee BM, Kacew S, Kim HS. 2007. Identification of differentially expressed genes in the testis of Sprague-Dawley rats treated with di(n-butyl) phthalate. Toxicology 234:103–112.

Schaefer CB, Ooi SK, Bestor TH, Bourc'his D. 2007. Epigenetic decisions in mammalian germ cells. Science 316:398–399.

Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3:1101–1108.

Sekaran S, Balaganapathy P, Parsanathan R, Elangovan S, Gunashekar J, Bhat F, Jagadeesan A. 2014. Lactational exposure of phthalate causes long-term disruption in testicular architecture by altering tight junctional and apoptotic protein expression in Sertoli cells of first filial generation pubertal Wistar rats. Hum Exp Toxicol doi: 10.1177/0960327114555926.

Shultz VD, Phillips S, Sar M, Foster PM, Gaido KW. 2001. Altered gene profiles in fetal rat testes after in utero exposure to di(n-butyl)phthalate. Toxicol Sci 64:233–242.

Skakkebaek NE, Rajpert-De Meyts E, Main KM. 2001. Testicular dysgenesis syndrome: An increasingly common developmental disorder with environmental aspects. Hum Reprod 16:972–978.

Soto AM, Vandenberg LN, Maffini MV, Sonnenschein C. 2008. Does breast cancer start in the womb? Basic Clin Pharmacol Toxicol 102:125–133.

Stocco DM. 2000. The role of the StAR protein in steroidogenesis: Challenges for the future. J Endocrinol 164:247–253.

Sugawara T, Saito M, Fujimoto S. 2000. Sp1 and SF-1 interact and cooperate in the regulation of human steroidogenic acute regulatory protein gene expression. Endocrinology 141:2895–2903.

Svechnikov K, Svechnikova I, Soder O. 2008. Inhibitory effects of mono-ethylhexyl phthalate on steroidogenesis in immature and adult rat Leydig cells in vitro. Reprod Toxicol 25:485–490.

Swan SH, Main KM, Liu F, Stewart SL, Kruse RL, Calafat AM, Mao CS, Redmon JB, Ternand CL, Sullivan S, Teague JL. 2005. Decrease in anogenital distance among male infants with prenatal phthalate exposure. Environ Health Perspect 113:1056–1061.

Val P, Lefrancois-Martinez AM, Veyssiere G, Martinez A. 2003. SF-1 a key player in the development and differentiation of steroidogenic tissues. Nucl Recept 1:8.

Vo TT, Jung EM, Dang VH, Jung K, Baek J, Choi KC, Jeung EB. 2009. Differential effects of flutamide and di-(2-ethylhexyl)phthalate on male reproductive organs in a rat model. J Reprod Dev 55:400–411.

Wu S, Zhu J, Li Y, Lin T, Gan L, Yuan X, Xiong J, Liu X, Xu M, Zhao D, Ma C, Li X, Wei G. 2010. Dynamic epigenetic changes involved in testicular toxicity induced by di-2-(ethylhexyl)phthalate in mice. Basic Clin Pharmacol Toxicol 106:118–123.

## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.