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Impact of Lactational Exposure to Polychlorinated Biphenyl Causes Epigenetic Modification and Impairs Sertoli Cells Functional Regulators in F_1 Progeny

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Abstract

Polychlorinated biphenyl (PCB) is an endocrine-disrupting chemical. Sertoli cells (SCs) provide physical and nutritional support for developing germ cells. Dysfunction in SCs has adverse effects on spermatogenesis. Previously, we found that the lactational exposure of PCBs (1, 2, and 5 mg/kg birth weight/day, orally from postnatal days 1 to 20) decreased the follicle-stimulating hormone receptor (FSHR) and androgen receptor (AR) expression in SCs of F_1 progeny. Transcription factors initiate and regulate the transcription of genes. DNA methylation plays an important role in epigenetic gene regulation. Hence, this study was aimed to identify the level of transcription factors regulating FSHR, AR gene expression, and DNA methylation in the promoter of these genes in SCs of both F_1 prepuberal and puberal offspring. DNA methylation in the promoter of FSHR and AR genes was examined by sodium bisulfite conversion technique. The protein levels of transcription factors (steroidogenic factor 1 [SF1], upstream stimulatory factors 1 and 2, c-fos, c-jun, and CREB-binding protein) and enzymes DNA methyltransferases (Dnmt1, Dnmt3ab, Dnmt3l, and histone deacetylase 1 [HDAC1]) were analyzed by Western blotting. The transcription factors that regulate the FSHR and AR gene in SCs were decreased in both the PCB-exposed F_1 progeny. Methylation was observed in the promoter of FSHR, AR, and SF1. The protein levels of Dnmt1, Dnmt3ab, Dnmt3l, and HDAC1 were increased in the PCBs-treated groups. Subsequently, it leads to transcriptional repression of the genes in SCs. Our finding suggests that PCBs caused epigenetic change in SCs, thereby it impaired SCs function in F_1 progeny.

Keywords

Sertoli cells, polychlorinated biphenyls, transcription factors, DNA methylation

Introduction

Sertoli cells (SCs) are somatic cells present within the seminiferous tubules and play an important role in the development of a functional testis. The SCs are required to support male germ cell development (spermatogenesis) in the mammalian testis. During development of the testis, the cessation of SCs proliferation and the onset of differentiation determine the final number of SCs, thereby it determines the level of male fertility.[1,2] Dysfunction in SCs may have adverse effects on spermatogenesis.[3] The exposure to endocrine disruptors (EDs) plays a key role on the epigenome shaping, thereby altering the endocrine function.[4] The EDs can affect the different levels of epigenetic control and in some cases can act transgenerationally, if the exposure to EDs occurs during the prenatal and early life.[5] DNA methylation is the addition of methyl group at fifth position of a cytosine residue within CpG dinucleotide motifs on both strands of DNA.[6] Unmethylated CpG islands are targets of transcription factors to start transcription. In contrast, the CpG sequences in inactive genes are usually methylated to suppress their expression.[7]

DNA methylation is catalyzed by DNA methyltransferases enzyme such as Dnmt1, Dnmt3ab and DNA methyltransferase 3-like (Dnmt3l), which transfer the methyl groups to the cytosine ring. Dnmt1 is involved in maintenance of methylation status during replication.[8] Dnmt3s (de novo DNA methyltransferases, including Dnmt3ab and the regulatory subunit DNMT3l) are responsible for the establishment of genomic DNA methylation patterns.[9] The Dnmt3l is genetically proven

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to be a necessary factor for DNA methylation of maternally imprinted genes and spermatogenesis.[10] Histones are core proteins of nucleosomes and acetylation of nuclear histones is regulated by histone acetyltransferase and histone deacetylase (HDAC). Histone acetyltransferase leads to the acetylation of core histone, enhances nucleosomal relaxation and subsequently induces transcription. The HDAC1 stabilizes nucleosomal structure and represses transcription.[11]

Polychlorinated biphenyls (PCBs) are widely present in the environment and cause potentially serious hazard to human and animal health.[12-14] The PCBs cross the placenta freely because of their lipophilicity and affect the developing fetus.[15-17] Prenatal PCB exposure can reduce the ratio of male to female births and also caused intelligence quotient deficits in the baby.[18] Prolonged time to pregnancy, reduced fecundity, developmental and cognitive deficits were observed in the children of mothers who had eaten the PCB-contaminated fish.[19,20] In rhesus monkeys, the exposure of PCBs is associated with alterations in the menstrual cycle, decreases fertility, increases spontaneous abortion and reduces conception rate.[21] Tabb and Blumberg[22] have found that PCB may alter the transcriptomic profile, particularly during development, and it also disrupts epigenetic mechanisms.[4] The pregnant rats exposed to PCB mixture throughout the period of gestation decreased the expression and activity of DNMTs in liver of the offspring.[23] Hence, the present study was focused to determine the epigenetic changes in SCs of both F_1 prepuberal and puberal rats.

Follicle-stimulating hormone receptor (FSHR) and testosterone play an important role in regulating the spermatogenesis process through its receptor present in the SCs. Transferrin is an iron carrier protein, which facilitates the availability of irons into the seminiferous tubules where it is essential for the development of germ cell. Previously, we have found that the lactational exposure of PCBs decreased the FSHR, androgen receptor (AR) and transferrin gene expression in SCs of both F_1 prepuberal and puberal offspring.[24] Transcription factors initiate and regulate the transcription of genes. DNA methylation plays an important role in epigenetic gene regulation. The present study was aimed to identify the protein level of transcription factors such as steroidogenic factor 1 (SF1), upstream stimulatory factor 1 (USF1), upstream stimulatory factor 2 (USF2), c-fos, c-jun, and CREB-binding protein (CBP) which regulate the FSHR, AR and transferrin gene expression and DNA methylation in the promoter of FSHR and AR genes in SCs of both prepuberal and puberal F_1 offspring. Then, the protein levels of DNA methyltransferases such as Dnmt1, Dnmt3ab, Dnmt3l, and HDAC1 were also examined in the SCs of F_1 progeny.

Materials and Methods

Chemicals

The PCBs sandy loam (Aroclor 1254, No CRM 913-50G, Lot No DG913), methylated and unmethylated primers, were purchased from Sigma-Aldrich Private Limited, Missouri, USA. Real-time polymerase chain reaction (RT-PCR) ready mix was purchased from KAPA, Washington, USA. Primary antibodies for USF1, USF2, c-fos, c-jun, CBP, Dnmt1, Dnmt3ab, SF1, Dnmt3l and HDAC1 were purchased from Santa Cruz Biotechnology, Texas, USA and Cell Signaling Technology, Danvers, USA. The secondary antibodies, horseradish peroxidase-conjugated rabbit–antimouse immunoglobulin G (IgG) and goat–antirabbit IgG were obtained from Genei (Bangalore, India). EZ DNA methylation kit was purchased from Zymo Research (Irvine, California). All other chemicals were purchased from Sisco Research Laboratories (Mumbai, India) of molecular and analytical grade.

Animal Care and Maintenance

Animals were maintained as per the national guidelines and protocols approved by the institutional animal ethical committee (no 01/04/2013). Nulliparous female albino rats (80 days old, Wistar strain [*Rattus norvegicus*], and weighing 180-200 g) with regular cyclicity were caged with male rats at a proportion of 2:1. The following day, rats were examined for the presence of vaginal plug. If mating was confirmed, the day was considered as embryonic day 1; each dam was placed in an individual cage and provided with water and food allowed to drink and eat ad libitum; dams were divided into 4 groups, each group consists of 6 animals. Group 1: control (corn oil alone as a vehicle), group 2: 1 mg PCBs (Aroclor 1254)/kg body weight (bw)/day, group 3: 2 mg PCBs (Aroclor 1254)/kg bw/day, and group 4: 5 mg PCBs (Aroclor 1254)/kg bw/day. The PCBs were dissolved in corn oil and administered daily through oral gavages to the lactating female rats from postnatal day (PND) 1 to PND 20. The male offspring from all the 4 groups were killed on both PND 21 and PND 60.

Isolation and Purification of SCs

The testes from both prepuberal and puberal F_1 rats were collected and SCs were isolated based on the procedure described by Majumdar et al[25] with some modification. The testicular tissues were chopped and sequentially digested with collagenase IV, collagenase I, and pancreatin with intermittent agitation. Then washed with DMEM medium by centrifuged at 800 rpm for 5 minutes, the final cell suspension was filtered through nylon mesh cell strainers (80 μm pore size). Purity of SCs was checked using oil red stain.[26] The isolated SCs were used to determine the protein level.

Immunoblotting

The protein levels were detected by Western blot analysis. The SCs were homogenized with radioimmunoprecipitation assay buffer and protease inhibitor cocktails. Protein concentration was estimated using bovine serum albumin as a standard.[27] Cell lysate (50 μg) was subjected to 10% to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, separated proteins on SDS-PAGE gels

Table 1. List of Primers used in Methylation-Specific PCR (MS-PCR).

Gene	Sense, Antisense Primer	Amplicon Size (bp)
FSHR—methylated forward	5′-AAAAAGGTATTTTTTGGTGGGTTAC-3′	208
Methylated reverse	5′-CAATAATAACATCCAAATCCCGTA-3′	
Unmethylated forward	5′-AAAAAGGTATTTTTTGGTGGGTTAT-3′	208
Unmethylated reverse	5′-CAATAATAACATCCAAATCCCATA-3′	
AR—methylated forward	5′-TTAAAGTTATTTTGCGTTAGTTTGC-3′	146
Methylated reverse	5′-CAACAATTTATAAATCGATCCCG-3′	
Unmethylated forward	5′-GTTTTTTAAAGTTATTTTGTGTTAGTTTGT-3′	144
Unmethylated reverse	5′-TACAACAATTTATAAATCAATCCCAC-3′	
SF1—methylated forward	5′-AATTAGTGTTTCGTTAATCGGAGGTC-3′	166
Methylated reverse	5′-GAAAAACCCGTCCTCTCACG-3′	
Unmethylated forward	5′-TAGTGTTTTGTTAATTGGAGGTTGT-3′	165
Unmethylated reverse	5′-CAAAAAACCCATCCTCTCACAC-3′	

Abbreviations: AR, androgen receptor; FSHR, follicle-stimulating hormone receptor; PCR, polymerase chain reaction; SF1, steroidogenic factor 1.

were transferred to polyvinylidene difluoride membrane (Millipore, USA). To block the nonspecific binding, the membranes were incubated with 5% skimmed milk for 2 hours. Membranes were immunoblotted with primary antibodies USF1, USF2, SF1, CBP, Dnmt1, Dnmt3ab, Dnmt3l, and HDAC1 (1:500 to 1:1000). The membranes were washed with Tris-buffered saline and incubated with horseradish peroxidase-labeled antimouse rabbit IgG or antirabbit mouse IgG antibody at a dilution of 1:10000. Protein bands were detected using enhanced chemiluminescence system kit and quantified in Chemi Doc XRS Imaging System (Bio-Rad, Hercules, California). The membranes were striped and reprobed for β-actin (1:1000) as an internal control.

Methylation-Specific PCR

The CpG islands in the promoter area of FSHR, AR, and SF1 were identified with a GC content of at least 50% using the Methprimer program. The Methyl Primer Express Software v1.0 (Applied Biosystems, USA) was used to design methylation-specific PCR (MS-PCR) primers, and the primers were listed in Table 1. Briefly, genomic DNA was extracted from SCs of both prepuberal and puberal F_1 progeny. The extracted DNA (500 ng) from animals of the control and experimental groups was subjected to bisulfite modification using the EZ DNA methylation kit (Zymo Research, Irvine, California). The bisulfite-modified DNA served as the template in MS-PCR. The PCR mix consisted of Master Mix Fast Hot Start Ready mix (KAPA), 0.2 μM primers, and 2 μL bisulfite-treated DNA in 20 μL total volume. The PCR conditions were as follows: preincubation at 95°C (3 minutes), 35 cycles of 15 seconds at 95°C denaturation, 15 seconds annealing at 55°C, and 1 minute extension at 72°C. The PCRs were performed with 2 primer pairs, which detected methylated and unmethylated DNA. After PCR, 10 μL of PCR mix was mixed with a loading dye and run on 2% agarose gel containing ethidium bromide. Stained gels were visualized and digitalized using the gel documentation system (Bio-Rad).

Statistical Analysis

All data were presented as mean (standard error of the mean) and analyzed by one-way analysis of variance followed by Students-Newman-Keuls test. Statistical analysis was performed by using the GraphPad Prism software (San Diego, California). Differences were considered statistically significant when the P level was less than .05.

Results

Impact of Lactational Exposure to PCBs on Transcription Factors in SCs of F_1 Progeny

Steroidogenic factor (SF1) in SCs is an important modulator of FSHR regulation by the gonadotropin hormones such as FSH and luteinizing hormone. The protein level of SF1 was decreased in a dose-dependent manner on SCs of F_1 prepuberal rats (Figure 1A), whereas in puberal rats, SF1 level was decreased in 2 and 5 mg PCBs-treated group, but there was no alteration observed between 1 mg PCBs treated and control (Figure 1B). The USF1 and USF2 bind and activate FSHR transcription through the proximal E box element, which is essential for activation of the FSHR promoter by SF1. The protein level of USF1 was gradually decreased in all the PCBs-treated groups of F_1 prepuberal offspring (Figure 2A), but in puberal rats, USF1 levels were decreased in 1 and 5 mg PCBs-treated groups and increased level was observed in the 2 mg PCBs-treated group than the control (Figure 2B).

The USF2 expression level was decreased only in 5 mg PCBs-treated group and it was increased in both 1 and 2 mg PCBs-treated groups of prepuberal offspring (Figure 3A). In case of puberal rats, there was no significant change observed in both 1 and 2 mg PCBs-treated groups when compared to control, but its level was decreased in the 5 mg PCBs-treated group alone (Figure 3B). c-jun and c-fos act as an AR coactivators. The protein level of c-jun was significantly decreased in a dose-dependent manner in prepuberal offspring (Figure 4A),

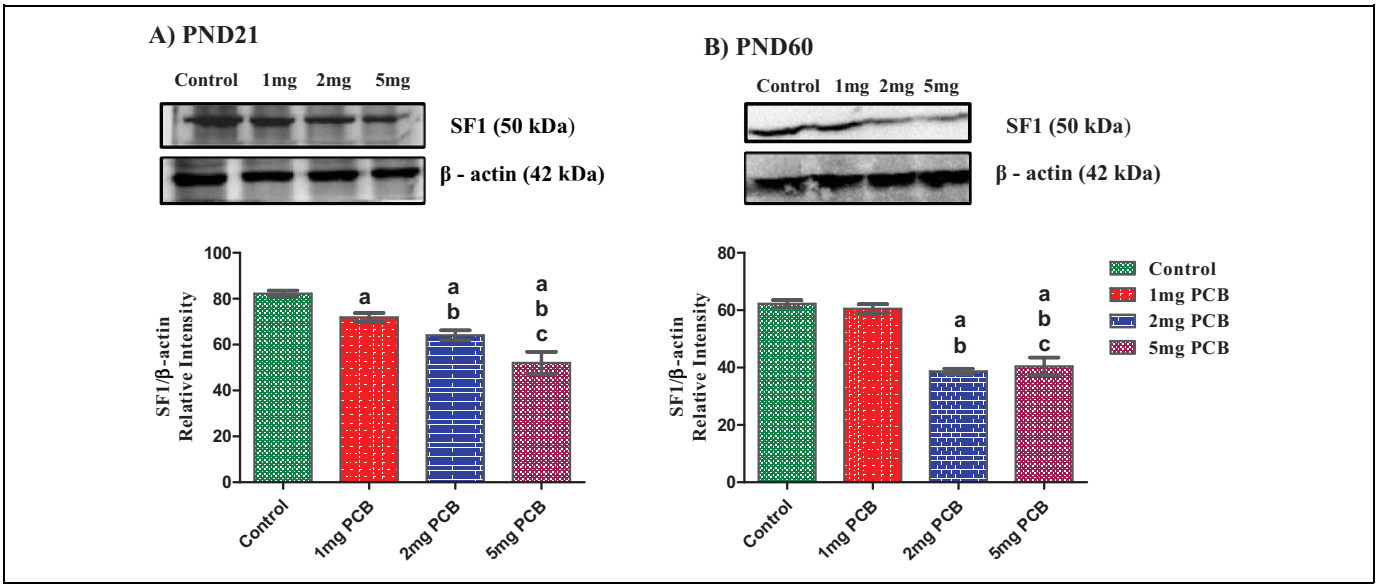

Figure 1. Effect of lactational exposure to PCBs on SF1 level in Sertoli cells of F_1 progeny. Each bar represents the mean (SEM) of 3 observations from pooled samples of 6 animals. (A) Control versus PCBs treated, (B) 1 mg PCBs versus 2 and 5 mg PCBs treated, and (C) 2 mg PCBs versus 5 mg PCBs treated at $P < .05$ level using Students-Newman-Keuls test. PCBs indicates polychlorinated biphenyl; SF1, steroidogenic factor 1; SEM, standard error of the mean.

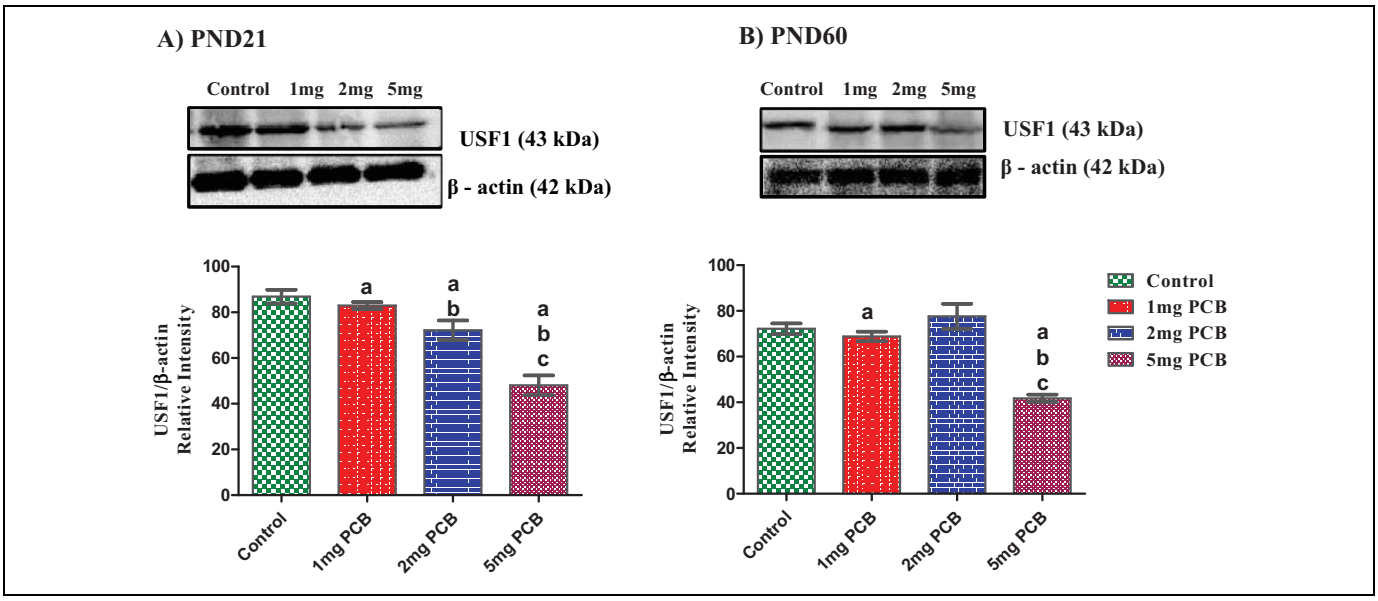

Figure 2. Effect of lactational exposure to PCBs on USF1 level in Sertoli cells of F_1 progeny. Each bar represents the mean (SEM) of 3 observations from pooled samples of 6 animals. (A) Control versus PCBs treated, (B) 1 mg PCBs versus 2 and 5 mg PCBs treated, and (C) 2 mg PCBs versus 5 mg PCBs treated at $P < .05$ level using Students-Newman-Keuls test. PCBs indicates polychlorinated biphenyl; SEM, standard error of the mean; USF1, upstream stimulatory factor 1.

whereas in puberal rats, c-jun protein level was decreased in 2 and 5 mg PCBs-treated group (Figure 4B).

The c-fos expression levels was decreased in 2 and 5 mg PCBs-treated group and there was no significant difference observed between 1 mg PCBs and control in both prepuberal and puberal F_1 offspring (Figure 5A and B). The CBP plays an essential role in regulating the transferrin gene expression in SCs. The CBP protein level was significantly decreased in all treated groups when compared to control (Figure 6A and B).

Impact of Lactational Exposure to PCBs on Promoter of FSHR, AR, and SF1 in SCs of F_1 Progeny

To screen the mechanism behind the decreased level of transcription factors (SF1, c-jun, c-fos, CBP, USF1 and USF2) in SCs of PCBs-treated groups, we explored the epigenetic modifications of FSHR, AR and SF1. The MS-PCR was used to screen the methylation changes in FSHR, AR, and SF1 in the SCs. Methylated bands were observed in the promoter of FSHR

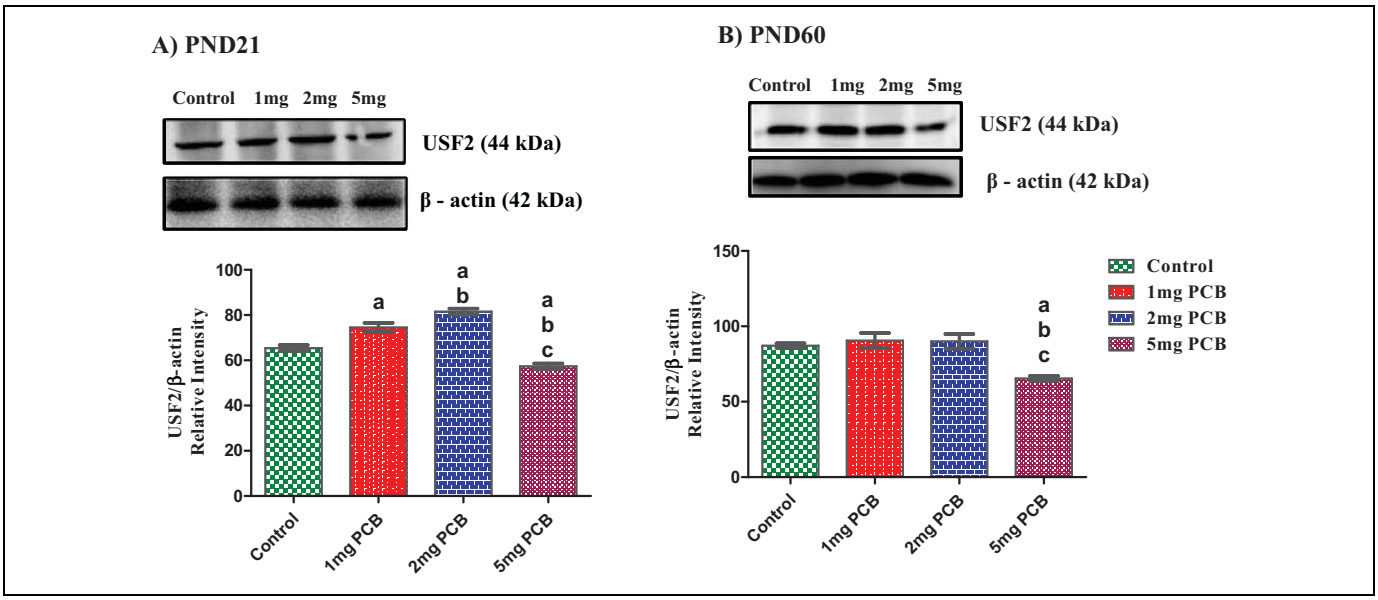

Figure 3. Effect of lactational exposure to PCB on USF2 level in Sertoli cells of F_1 progeny. Each bar represents the mean (SEM) of 3 observations from pooled samples of 6 animals. (A) Control versus PCBs treated, (B) 1 mg PCBs versus 2 and 5 mg PCBs treated, and (C) 2 mg PCBs versus 5 mg PCBs treated at $P < .05$ level using Students-Newman-Keuls test. PCBs indicates polychlorinated biphenyl; SF1, steroidogenic factor 1; SEM, standard error of the mean USF2, upstream stimulatory factor 2.

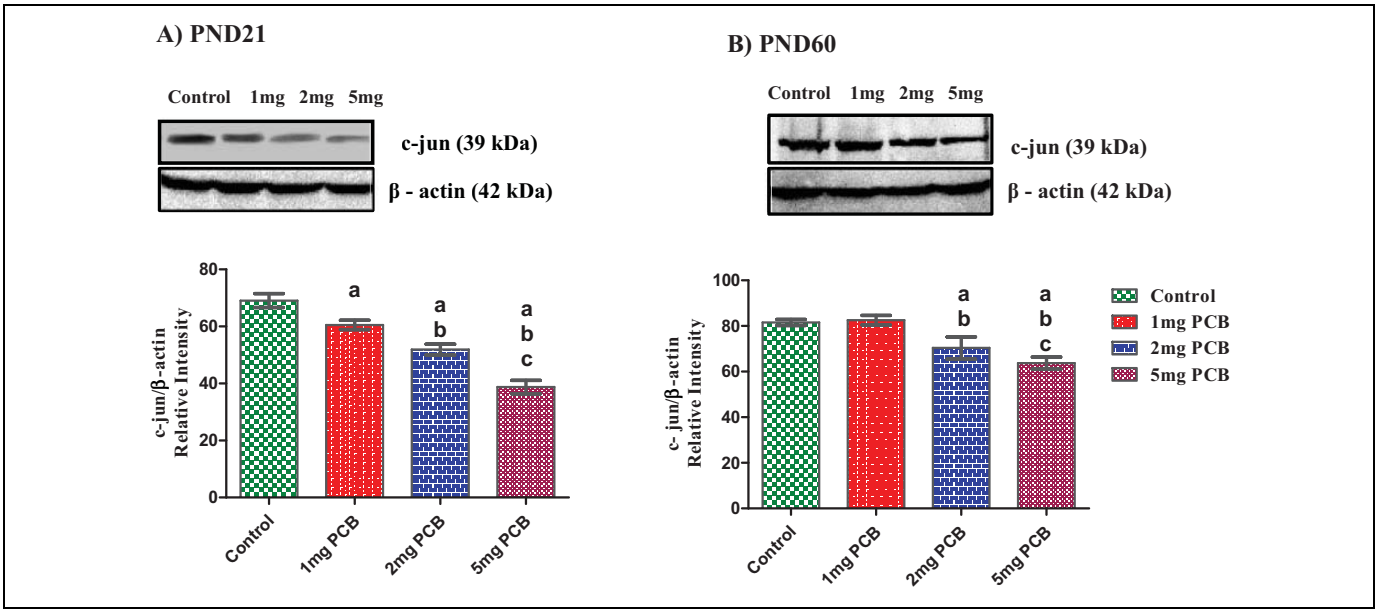

Figure 4. Effect of lactational exposure to PCBs on c-jun level in Sertoli cells of F_1 progeny. Each bar represents the mean (SEM) of 3 observations from pooled samples of 6 animals. (A) Control versus PCBs treated, (B) 1 mg PCBs versus 2 and 5 mg PCBs treated, and (C) 2 mg PCBs versus 5 mg PCBs treated at $P < .05$ level using Students-Newman-Keuls test. PCBs indicates polychlorinated biphenyl; SEM, standard error of the mean.

and AR (Figures 7A and B and 8A and B) in both the F_1 progeny. Likewise, methylated bands were observed in the promoter of SF1 in the SCs of PCBs-treated groups in F_1 pre-puberal rats (Figure 9A); in contrast, methylation was observed only in the 2 mg PCBs-treated groups and unmethylated bands was observed in all the PCBs-treated groups in puberal off-spring (Figure 9B).

Impact of Lactational Exposure to PCBs on DNA Methyltransferases (Dnmt1, Dnmt3ab, and Dnmt3l) and HDAC1 in SCs of F_1 Progeny

Dnmt1 is required for the maintenance of methylation after DNA replication, whereas Dnmt3ab will initiate the methylation along with its regulator Dnmt3l. The protein expression level of Dnmt1

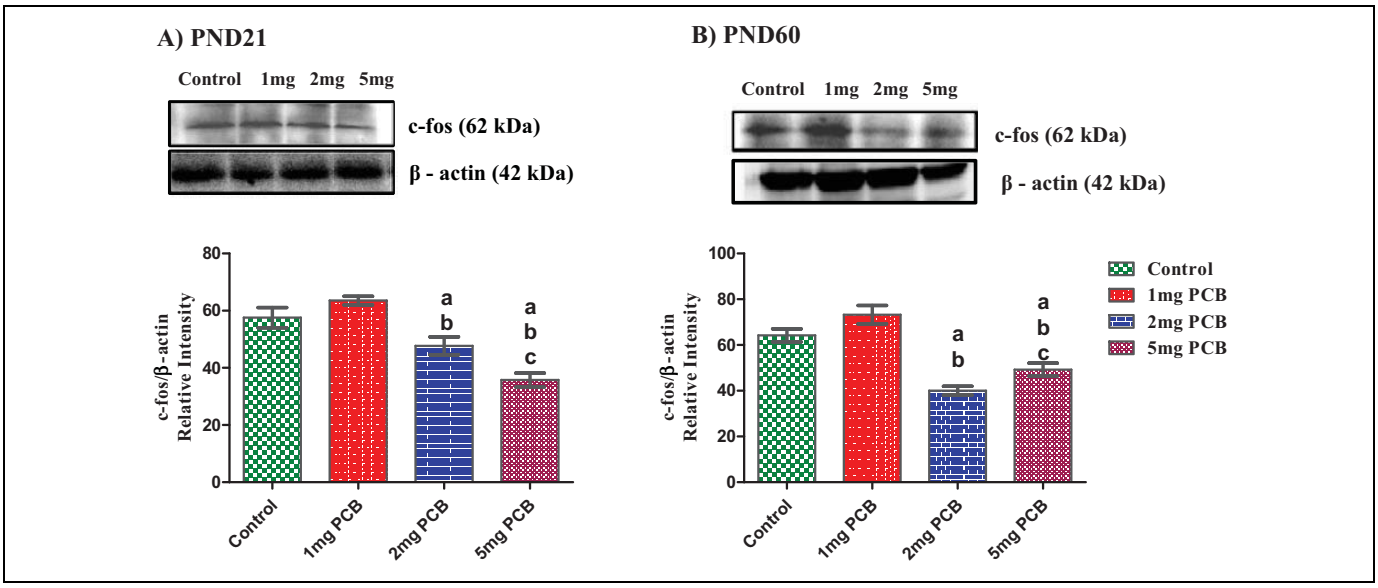

Figure 5. Effect of lactational exposure to PCBs on c-fos level in Sertoli cells of F_1 progeny. Each bar represents the mean (SEM) of 3 observations from pooled samples of 6 animals. (A) Control versus PCBs treated, (B) 1 mg PCBs versus 2 and 5 mg PCBs treated, and (C) 2 mg PCBs versus 5 mg PCBs treated at $P < .05$ level using Students-Newman-Keuls test. PCBs indicates polychlorinated biphenyl; SEM, standard error of the mean.

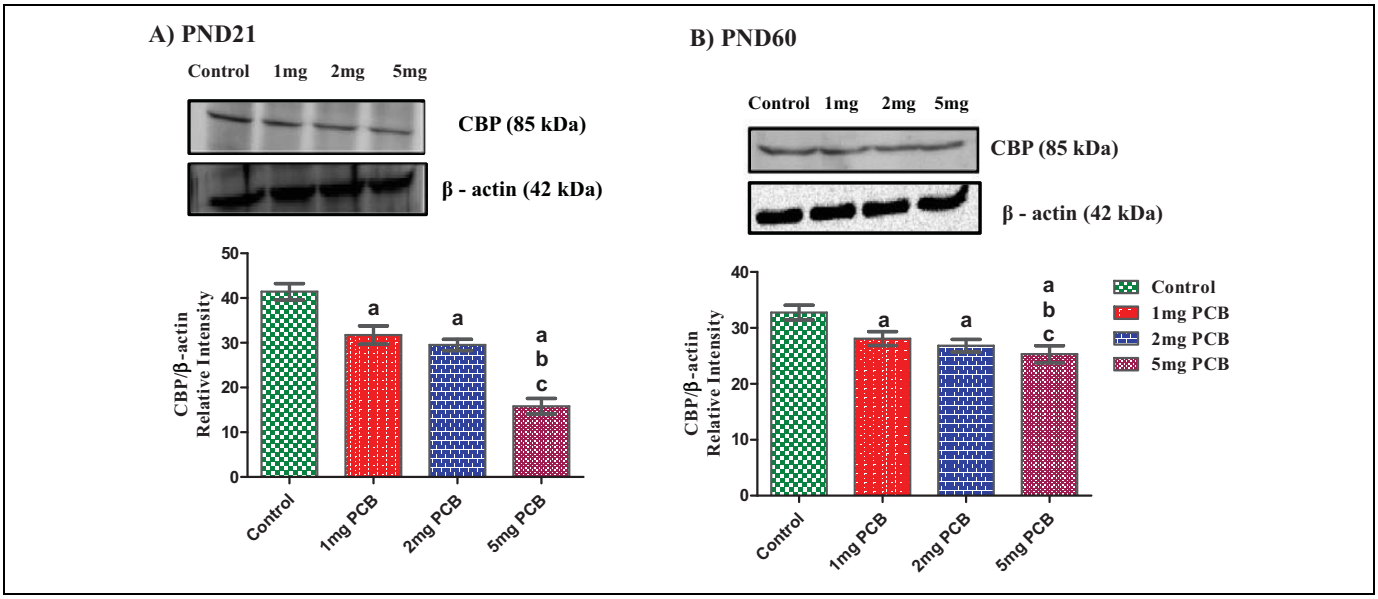

Figure 6. Effect of lactational exposure to PCBs on CBP level in Sertoli cells of F_1 progeny. Each bar represents the mean (SEM) of 3 observations from pooled samples of 6 animals. (A) Control versus PCBs treated, (B) 1 mg PCBs versus 2 and 5 mg PCBs treated, and (C) 2 mg PCBs versus 5 mg PCBs treated at $P < .05$ level using Students-Newman-Keuls test. PCBs indicates polychlorinated biphenyl; CBP, CREB-binding protein; SEM, standard error of the mean.

was increased in a dose-dependent manner in all PCBs-treated groups of F_1 prepuberal rats (Figure 10A). Whereas in puberal rats, the Dnmt1 expression level was increased in 1 and 2 mg PCBs-treated groups. Dnmt1 level was decreased in 5 mg PCBs-treated group than 1 and 2 mg PCBs-treated groups of F_1 puberal rats (Figure 10B). Dnmt3ab expression level was increased in the 2 and 5 mg PCBs-treated groups than the control, but there was no significant difference observed between control and 1 mg PCBs-treated group of both F_1 progeny (Figure 11A and B). Dnmt3l protein expression level was also increased in all the PCBs-treated groups in prepuberal offspring (Figure 12A) and its level was increased in all the PCBs-treated group than the control, but there was no difference observed between 2 and 5 mg PCBs-treated group (Figure 12A and B). The HDACs remove acetyl group and suppress the gene expression. In this study, HDAC1 expression level was increased in 2 and 5 mg

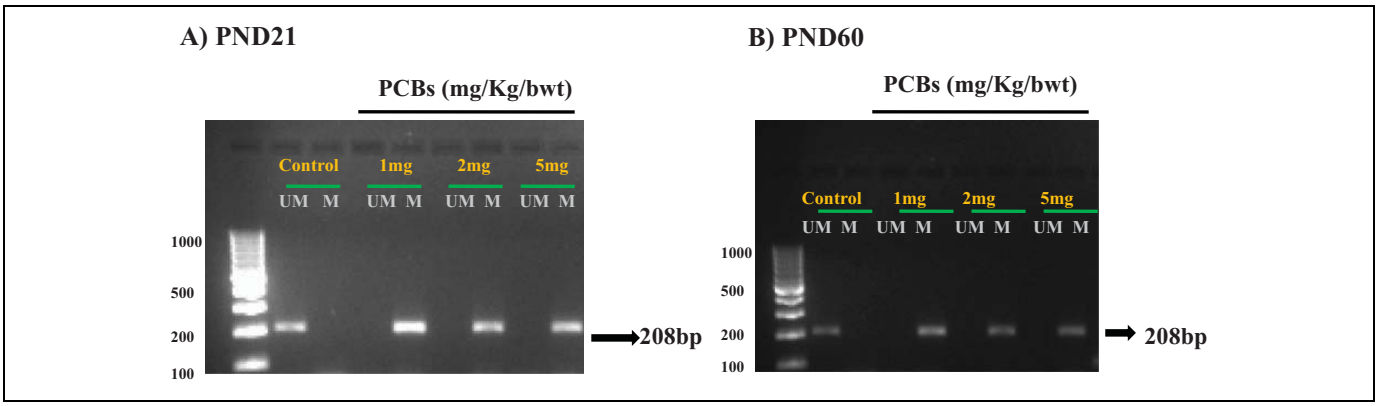

Figure 7. Effect of lactational exposure to PCBs on methylation of FSHR promoter in Sertoli cells of F_1 progeny. Each bar represents the mean (SEM) of 3 observations from pooled samples of 6 animals. PCBs indicates polychlorinated biphenyl; FSHR, follicle-stimulating hormone; SEM, standard error of the mean.

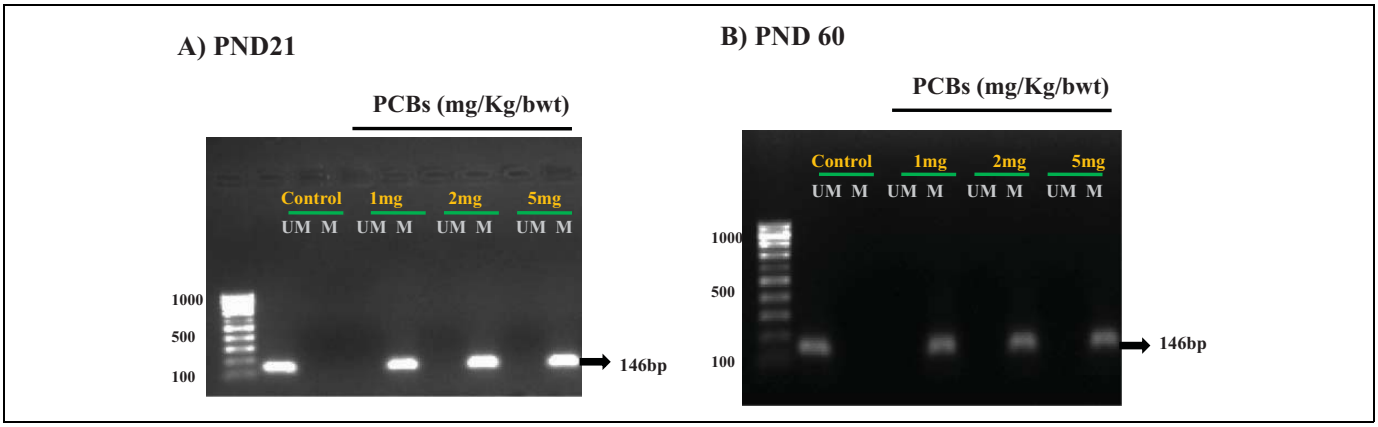

Figure 8. Effect of lactational exposure to PCBs on methylation of AR promoter in Sertoli cells of F_1 progeny. Each bar represents the mean (SEM) of 3 observations from pooled samples of 6 animals. PCBs indicates polychlorinated biphenyl; AR, androgen receptor; SEM, standard error of the mean.

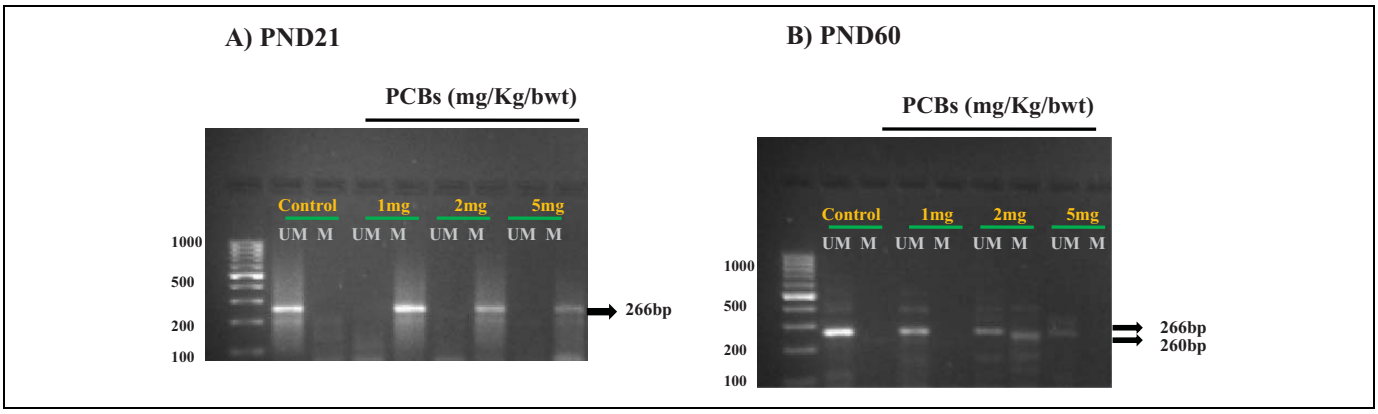

Figure 9. Effect of lactational exposure to PCBs on methylation of SF1 in Sertoli cells of F_1 progeny. Each bar represents the mean (SEM) of 3 observations from pooled samples of 6 animals. PCBs indicates polychlorinated biphenyl; SF1, steroidogenic factor 1; SEM, standard error of the mean.

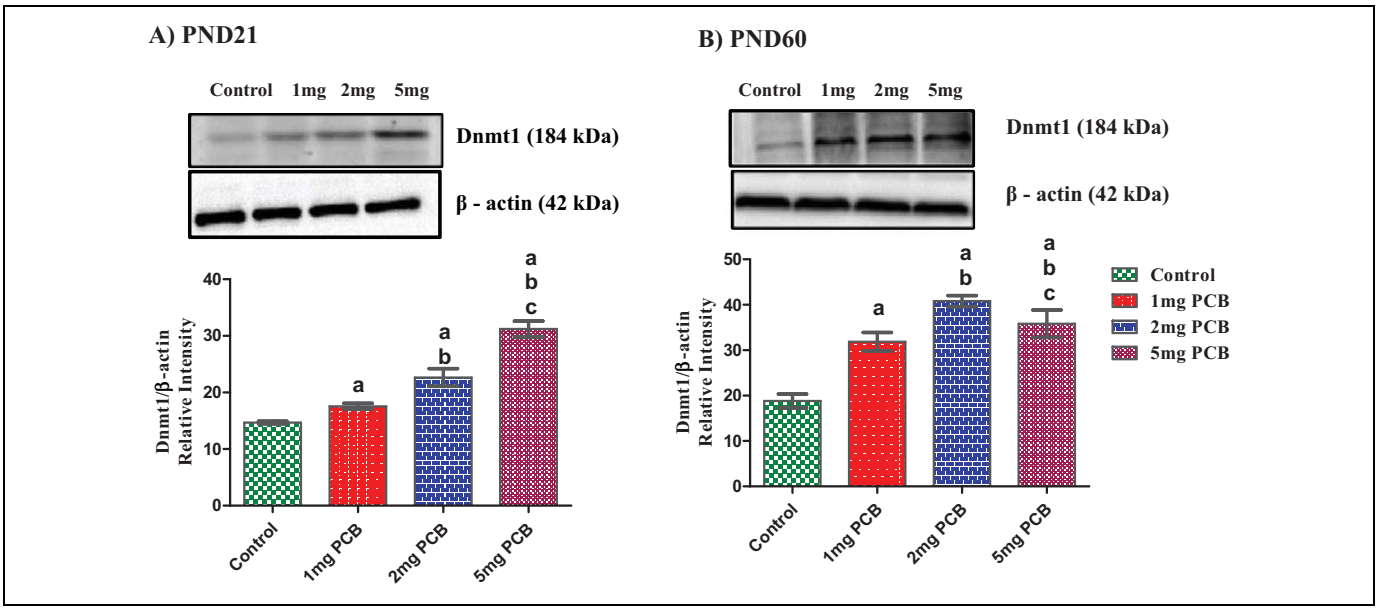

Figure 10. Effect of lactational exposure to PCBs on Dnmt1 level in Sertoli cells of F_1 progeny. Each bar represents the mean (SEM) of 3 observations from pooled samples of 6 animals. (A) Control versus PCBs treated, (B) 1 mg PCBs versus 2 and 5 mg PCBs treated, and (C) 2 mg PCBs versus 5 mg PCBs at $P < .05$ level using Students-Newman-Keuls test. PCBs indicates polychlorinated biphenyl; SEM, standard error of the mean.

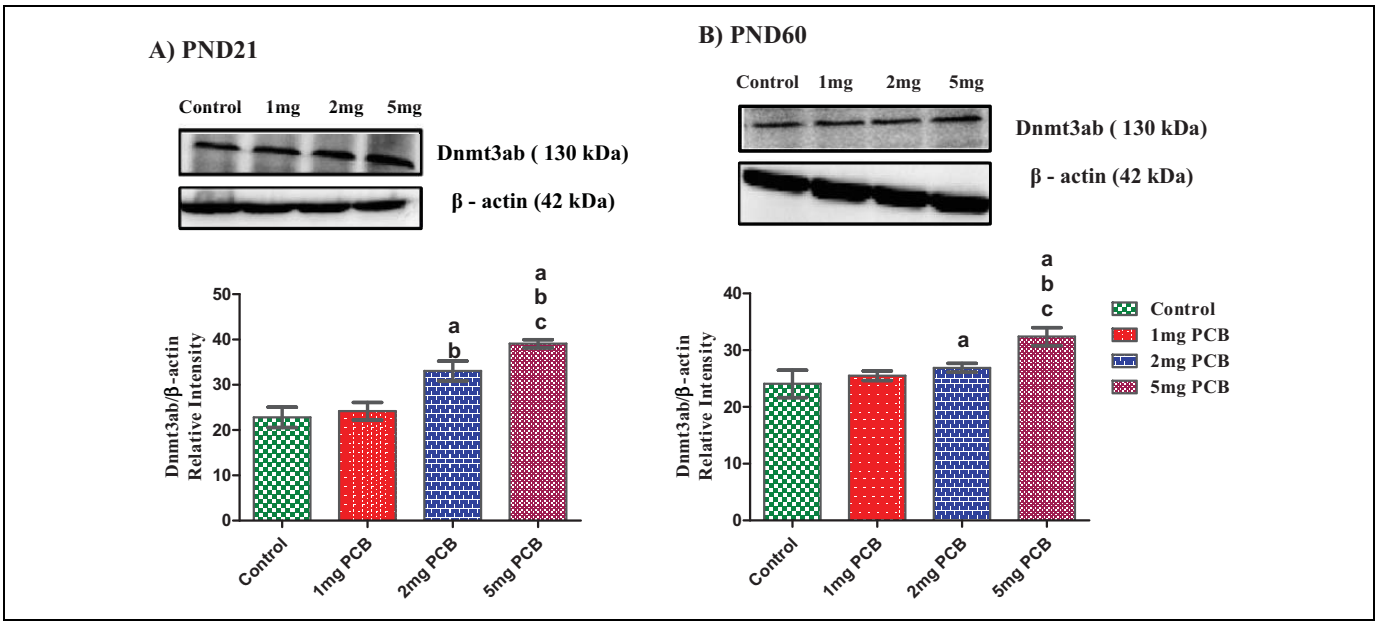

Figure 11. Effect of lactational exposure to PCBs on Dnmt3ab level in Sertoli cells of F_1 progeny. Each bar represents the mean (SEM) of 3 observations from pooled samples of 6 animals. (A) Control versus PCBs treated, (B) 1 mg PCBs versus 2 and 5 mg PCBs, and (C) 2 mg PCBs versus 5 mg PCBs treated at $P < .05$ level using Students-Newman-Keuls test. PCBs indicates polychlorinated biphenyl; SEM, standard error of the mean.

PCBs-treated group than the control, but there was no significant difference observed between control and 1 mg PCBs-treated group in F_1 puberal offspring (Figure 13A and B).

Discussion

An earlier study from our laboratory demonstrated that PCBs exposure affects the expression of luteinizing hormone receptor (LHR) and steroidogenic regulating enzymes, thereby it disrupts the steroidogenic pathway in adult rats.[12,13] Krishnamoorthy et al[28] have shown that PCB exposure decreased the FSHR, AR and connexin 43 expression in SCs of adult rats. Lactational exposure of PCBs affects the testicular histoarchitecture, testosterone, ABP and estradiol levels in both serum and testicular fluid, which is important for the germ cells development.[24] Our earlier studies have demonstrated that lactational

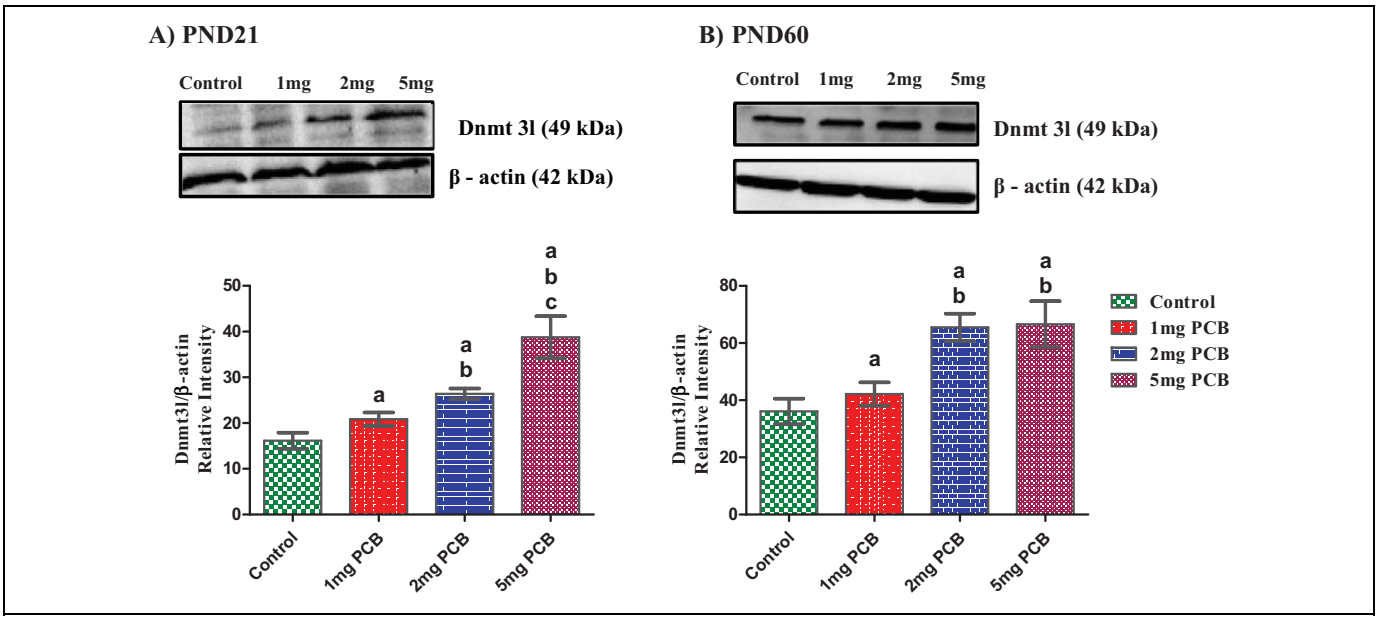

Figure 12. Effect of lactational exposure to PCBs on Dnmt3l level in Sertoli cells of F_1 progeny. Each bar represents the mean (SEM) of 3 observations from pooled samples of 6 animals. (A) Control versus PCBs treated, (B) 1 mg PCBs versus 2 and 5 mg PCBs treated, and (C) 2 mg PCBs versus 5 mg PCBs treated at $P < .05$ level using Students-Newman-Keuls test. PCBs indicates polychlorinated biphenyl; SEM, standard error of the mean.

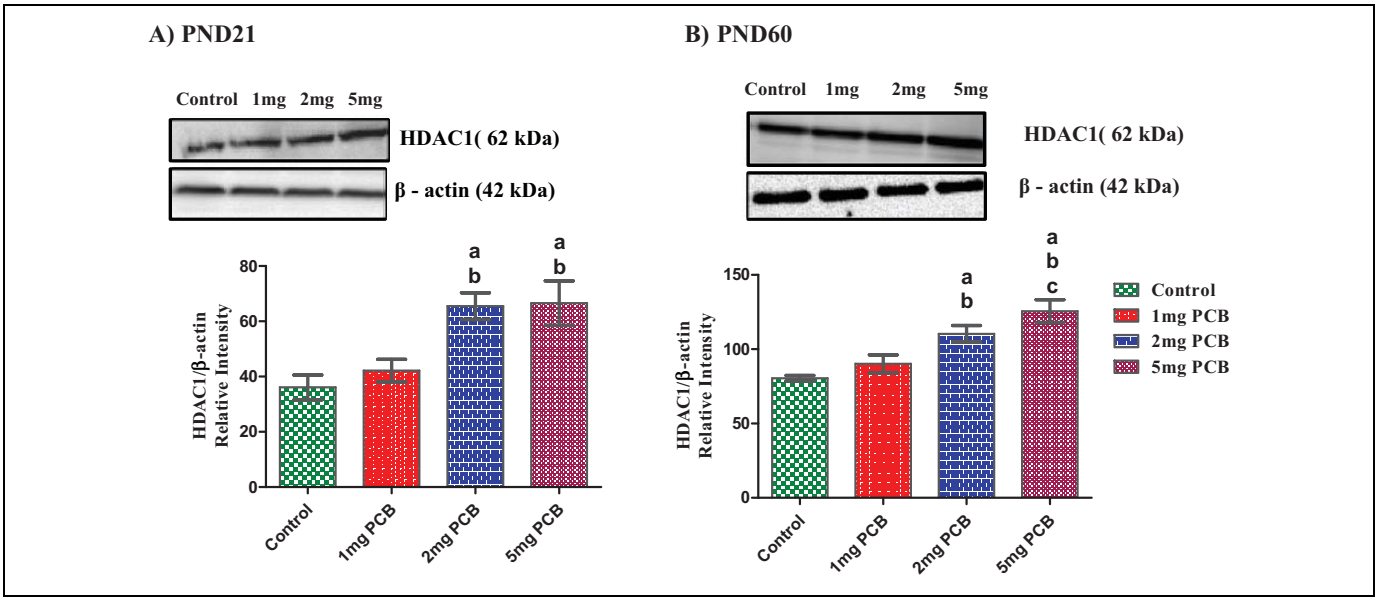

Figure 13. Effect of lactational exposure to PCBs on HDAC1 level in Sertoli cells of F_1 progeny. Each bar represents the mean (SEM) of 3 observations from pooled samples of 6 animals. (A) Control versus PCBs treated, (B) 1 mg PCBs versus 2 and 5 mg PCBs, and (C) 2 mg PCBs versus 5 mg PCBs treated at $P < .05$ level using Students-Newman-Keuls test. PCBs indicates polychlorinated biphenyl; HDAC1, histone deacetylase 1; SEM, standard error of the mean.

exposure of PCB decreased the expression levels of FSHR, AR and transferrin in SCs of both F_1 prepuberal and puberal offspring.[24] To continue with, the present study was focused to determine the molecular mechanism behind the decreased level of FSHR, AR and transferrin level in SCs by determining the level of transcription factors involved in it and DNA methylation pattern in the promoter region.

The FSH acts through FSHR present in SCs and thus it regulates SCs proliferation and functions of SCs secreted protein.[29] Methylation in the promoter regions leads to inhibition of the binding of transcription factors directly or it inhibits the recruitment of proteins which helps in binding of the transcription factors to their cognate cis elements.[30] In addition, methylated promoter regions are often coupled with regional histone

deacetylation, suggesting that these 2 mechanisms may act in concert to accomplish transcriptional silencing.[11] Transcriptional regulation of SCs differentiation is in part controlled through the binding of specific transcription factors to response elements within these genes promoters. E box sequence (CACGTG, -124/-119) within the FSHR promoter has been identified as an essential positive regulatory element that binds with the ubiquitous transcription factors such as USF1 and USF2.[31]

Steroidogenic factor 1 (SF1) activates FSHR expression by directly binding to the E box response element in FSHR promoter region. The USF1 and USF2 are DNA-binding proteins which act as a coregulator of SF1 in FSHR transcription.[32] Griswold and Kim[33] have found that methylation of a CpG sequence within a consensus E box element decreased the binding affinity of USF1 and USF2 transcription factors for this element. In the present study, lactational exposure of PCBs resulted DNA methylation in the E box element of FSHR promoter, so this may affect the binding affinity of USF1 and USF2. In order to confirm this, the protein levels of SF1, USF1, and USF2 in SCs of F_1 offspring were investigated. The protein levels of SF1 were decreased in a dose-dependent manner; whereas USF1 and USF2 were decreased significantly in 5 mg PCBs-treated groups of F_1 offspring. DNA methylation was observed in the promoter of SF1 in SCs of PCBs-treated groups in both F_1 prepuberal and puberal rats. Wu et al[34] have studied that di-(2-ethylhexyl) phthalate (DEHP) exposure significantly increased DNA methylation levels on GD19 and PND3. Further, they have also shown that DNA methyltransferases levels were significantly increased in the testis and concluded that the changes in DNA methylation are mediated by the DNA methyltransferases. Sekaran and Jagadeesan[35] have found that gestational exposure of DEHP induced hypermethylation in SF1 and SP1 promoter in Leydig cell, thereby it affects the Leydig cell function in F_1 offspring. This suggests that methylation in the promoter of FSHR, SF1 alters the expression level of USF1/2 and SF1, thereby it represses the FSHR expression in SCs of PCBs-exposed F_1 progeny.

Transferrin gene expression has been regulated by the binding and interactions of basic helix-loop-helix (bHLH) and cAMP response element binding protein (CREB) to an E box and cyclic AMP response element (CRE), respectively. Interaction between the bHLH and CREB is facilitated through subsequent binding of CBP)/p300.[36] In this study, CBP protein level was decreased in all the PCBs-treated groups on SCs of both the F_1 progeny. This depicts that the decreased expression level of CBP in SCs may fail to interact with the bHLH and CREB in the promoter of transferrin gene and this may be the one of the reasons for the decreased level of transferrin observed in the SCs of both F_1 prepuberal and puberal offspring.

The AR plays an important role in the germ cell development and maintenance of spermatogenesis. Absence of AR in SCs leads to hypoplasia of seminiferous tubules and absence of germ cell differentiation.[37] Previously, we have found that the lactational exposure of PCBs decreased the AR protein level in SCs of both prepuberal and puberal F_1 offspring.[24] To identify the mechanism behind the decreased level of AR in SCs, we have checked the DNA methylation pattern in the promoter of AR and level of transcription factors (activated protein-1 [AP1] complex) regulating AR gene expression in SCs. The AP1 transcription factor complex consists of a dimer between members of the c-fos, c-jun and activating transcription factor (ATF) families of proteins. c-fos proteins can only heterodimerize, while the c-jun proteins form both homo- and heterodimerize with fos; thus, it forms transcriptionally active complexes. The heterodimer of c-jun and c-fos preferentially binds to the TRE (12-O-Tetradecanoylphorbol-13-acetate response element) in the promoter of AR and initiate the transcription.[38,39] c-jun acts as an AR coactivator binding to the *N*-terminal domain at amino acids 503 to 555,[40] promoting AR dimerization and gene transcription.[41] In the present study, lactational exposure of PCBs decreased the expression levels of c-jun, c-fos in 2 and 5 mg PCBs-treated groups on SCs of both F_1 progeny and DNA methylation was observed in all the PCB-treated groups of both prepuberal and puberal F_1 offspring. This suggests that the decreased expression levels of c-fos, c-jun and methylation in the promoter of AR may be the reason for the decreased level of AR gene expression in SCs of F_1 progeny.

Then we would like to explore the level of DNA methyltransferases (Dnmt1, Dnmt3ab, Dnmt3l) which catalyses the DNA methylation, whereas HDAC1 catalyses the histone deacetylation and we found that Dnmt1, Dnmt3ab and Dnmt3l levels were increased in all the PCBs-treated groups in SCs of both F_1 offspring. The increased level of DNA methyltransferases is responsible for the methylation observed in the promoter of FSHR, AR and SF1. Sekaran and Jagadeesan[35] have also found that increased protein levels of Dnmt1 and Dnmt3ab altered the methylation pattern in SF1 and SP1 gene in Leydig cells of DEHP-exposed F_1 offspring. Histone acetylation is important for initiating and maintaining transcriptionally active genes and histone deacetylation requisite for gene silencing. To support with, previous studies have shown that AR level was downregulated by HDAC1 in a deacetylase-dependent manner.[42,43] In this study, HDAC1 protein level was increased in a dose-dependent manner and this increased expression of HDAC1 removes the acetyl group from the histone complex, thereby it represses the gene expression in the PCBs-treated groups.

Casati et al[44] reviewed that PCBs affect the histone post-translational modifications in a dimorphic way possibly as the result of an alteration in gene expression of the enzymes involved in histone modification. Although MS-PCR is not a quantitative method to measure the DNA methylation, further studies are warranted to sequence the PCR end products of bisulfite-treated DNA. In conclusion, the present study found that the lactational exposure of PCBs affects the SCs functional regulators in a dose-dependent manner through epigenetic-mediated mechanism.

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References

1. Orth JM, Gunsalus GL, Lamperti AA. Evidence from Sertoli cell-depleted rats indicates that spermatid number in adults depends on numbers of Sertoli cells produced during perinatal development. *Endocrinology*. 1988;122(3):787-794.
2. Sharpe RM, McKinnell C, Kivlin C, Fisher JS. Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. *Reproduction*. 2003;125(6):769-784.
3. Choi MS, Park HJ, Oh JH, Lee EH, Park SM, Yoon S. Nonylphenol-induced apoptotic cell death in mouse TM4 Sertoli cells via the generation of reactive oxygen species and activation of the ERK signaling pathway. *J Appl Toxicol*. 2014;34(6):628-636.
4. Casati L, Sendra R, Poletti A, Negri-Cesi P, Celotti F. Androgen receptor activation by polychlorinated biphenyls: epigenetic effects mediated by the histone demethylase Jarid1b. *Epigenetics*. 2013;8(10):1061-1068.
5. Chuang JC, Jones PA. Epigenetics and microRNAs. *Pediatr Res*. 2007;61(5 pt 2):24R-29R.
6. Kafri T, Gao X, Razin A. Mechanistic aspects of genome-wide demethylation in the preimplantation mouse embryo. *Proc Natl Acad Sci U S A*. 1993;90(22):10558-10562.
7. Belzil VV, Bauer PO, Gendron TF, Murray ME, Dickson D, Petrucelli L. Characterization of DNA hypermethylation in the cerebellum of c9FTD/ALS patients. *Brain Res*. 2014;1584:15-21.
8. Reik W, Dean W, Walter J. Epigenetic reprogramming in mammalian development. *Science*. 2001;293(5532):1089-1093.
9. Lechner M, Boshoff C, Beck S. Cancer epigenome. *Adv Genet*. 2010;70:247-276.
10. Hata K, Okano M, Lei H, Li E. Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. *Development*. 2002;129(8):1983-1993.
11. Ng HH, Bird A. DNA methylation and chromatin modification. *Curr Opin Genet Dev*. 1999;9(2):158-163.
12. Murugesan P, Muthusamy T, Balasubramanian K, Arunakaran J. Polychlorinated biphenyl (Aroclor 1254) inhibits testosterone biosynthesis and antioxidant enzymes in cultured rat Leydig cells. *Reprod Toxicol*. 2008; 25(4):447-454.
13. Murugesan P, Senthilkumar J, Balasubramanian K, Aruldhas MM, Arunakaran J. Impact of polychlorinated biphenyl Aroclor 1254 on testicular antioxidant system in adult rats. *Hum Exp Toxicol*. 2005;24(2):61-66.
14. Colciago A, Casati L, Mornati O, et al. Chronic treatment with polychlorinated biphenyls (PCB) during pregnancy and lactation in the rat. Part 2: effects on reproductive parameters, on sex behavior, on memory retention and on hypothalamic expression of aromatase and 5alpha-reductases in the offspring. *Toxicol Appl Pharmacol*. 2009;239(1):46-54.
15. Jacobson JL, Jacobson SW. *Developmental Effects of PCBs in the Fish Eater Cohort Studies. PCBs—Recent Advances in the Environmental Toxicology and Health Effects of PCBs*. Lexington, KY: The University Press of Kentucky; 2001:127-136.
16. Odland J, Deutch B, Hansen JC, Burkow IC. The importance of diet on exposure to and effect of persistent organic pollutants on human health in the Arctic. *Acta Paediatr*. 2003;92(11):1255-1266.
17. Sathish Kumar T, Sugantha Priya E, Raja Singh P, Arunakaran J. Lactational exposure of polychlorinated biphenyls downregulates critical genes in Leydig cells of F1 male progeny (PND21) [Published online October 26, 2016]. *Andrologia*. doi:10.1111/and.12734.
18. Hertz-Picciotto I, Jusko TA, Willman EJ, et al. A cohort study of in utero polychlorinated biphenyl (PCB) exposures in relation to secondary sex ratio. *Environ Health*. 2008;7(1):1-8.
19. Buck GM, Vena JE, Schisterman EF, et al. Parental consumption of contaminated sport fish from Lake Ontario and predicted fecundability. *Epidemiology*. 2000;11(4):388-393.
20. Wu JP, Luo XJ, Zhang Y, et al. Bioaccumulation of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) in wild aquatic species from an electronic waste (e-waste) recycling site in South China. *Environ Int*. 2008;34(8):1109-1113.
21. Arnold DL, Mes J, Bryce F, et al. A pilot study on the effects of Aroclor 1254 ingestion by rhesus and cynomolgus monkeys as a model for human ingestion of PCBs. *Food Chem Toxicol*. 1990;28(12):847-857.
22. Tabb MM, Blumberg B. New modes of action for endocrine disrupting chemicals. *Mol Endocrinol*. 2006;20(3):475-482.
23. Desaulniers D, Xiao GH, Lian H, et al. Effects of mixtures of polychlorinated biphenyls, methylmercury, and organochlorine pesticides on hepatic DNA methylation in prepubertal female Sprague-Dawley rats. *Int J Toxicol*. 2009;28(4):294-307.
24. Sugantha Priya E, Sathish Kumar T, Balaji S, et al. Lactational exposure effect of polychlorinated biphenyl on rat Sertoli cell markers and functional regulators in prepuberal and puberal F_1 offspring. *J Endocrinol Invest*. 2016;40(1);91-100.
25. Majumdar SS, Tsuruta J, Griswold MD, Bartke A. Isolation and culture of Sertoli cells from the testes of adult Siberian hamsters: analysis of proteins synthesized and secreted by Sertoli cells cultured from hamsters raised in a long or a short photoperiod. *Biol Reprod*. 1995;52(3):658-666.
26. Raychoudhury S, Thompson EW, Blackshaw A, Irving MG. Sertoli cells as paracrine modulators of DNA synthesis in rat peritubular myoid cells in culture. *J Reprod Fertil*. 1993;99(2):513-518.

27. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951; 193(1):265-275.
28. Krishnamoorthy G, Selvakumar K, Venkataraman P, Elumalai P, Arunakaran J. Lycopene supplementation prevents reactive oxygen species mediated apoptosis in Sertoli cells of adult albino rats exposed to polychlorinated biphenyls. *Interdiscip Toxicol*. 2013; 6(2):83-92.
29. Skinner MK, Schlitz SM, Anthony CT. Regulation of Sertoli cell differentiated function: testicular transferrin and androgen-binding protein expression. *Endocrinology*. 1989;124(6): 3015-3024.
30. Jones PL, Veenstra GJ, Wade PA, et al. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat Genet*. 1998;19(2):187-191.
31. Heckert LL, Daggett MA, Chen J. Multiple promoter elements contribute to activity of the follicle-stimulating hormone receptor (FSHR) gene in testicular Sertoli cells. *Mol Endocrinol*. 1998; 12(10):1499-1512.
32. Heckert LL. Activation of the rat follicle-stimulating hormone receptor promoter by steroidogenic factor 1 is blocked by protein kinase a and requires upstream stimulatory factor binding to a proximal E box element. *Mol Endocrinol*. 2001;15(5): 704-715.
33. Griswold MD, Kim JS. Site-specific methylation of the promoter alters deoxyribonucleic acid-protein interactions and prevents follicle-stimulating hormone receptor gene transcription. *Biol Reprod*. 2001;64(2):602-610.
34. Wu S, Zhu J, Li Y, et al. Dynamic epigenetic changes involved in testicular toxicity induced by di-2-(ethylhexyl) phthalate in mice. *Basic Clin Pharmacol Toxicol*. 2010;106(2):118-123.
35. Sekaran S, Jagadeesan A. In utero exposure to phthalate down-regulates critical genes in Leydig cells of F1 male progeny. *J Cell Biochem*. 2015;116(7):1466-1477.
36. Chaudhary J, Skinner MK. E-box and cyclic adenosine monophosphate response elements are both required for follicle-stimulating hormone-induced transferrin promoter activation in Sertoli cells. *Endocrinology*. 1999;140(3):1262-1271.
37. Regadera J, Martinez-Garcia F, Gonzalez-Peramatao P, Serrano A, Nistal M, Suárez-Quian C. Androgen receptor expression in Sertoli cells as a function of seminiferous tubule maturation in the human cryptorchid testis. *J Clin Endocrinol Metab*. 2001;86(1):413-421.
38. Karin M, Liu Z, Zandi E. AP1 function and regulation. *Curr Opin Cell Biol*. 1997:9(2):240-246.
39. O'Shea EK, Rutkowski R, Kim PS. Mechanism of specificity in the Fos-jun oncoprotein heterodimer. *Cell*. 1992;68(4):699-708.
40. Wise SC, Burmeister L A, Zhou XF, et al. Identification of domains of c-Jun mediating androgen receptor transactivation. *Oncogene*. 1998;16(15):2001-2010.
41. Bubulya A, Chen SY, Fisher CJ, Zheng Z, Shen XQ, Shemshedini L. c-Jun potentiates the functional interaction between the amino and carboxyl termini of the androgen receptor. *J Biol Chem*. 2001; 276(48):44704-44711.
42. Gaughan L, Logan IR, Cook S, Neal DE, Robson CN. Tip60 and histone deacetylase 1 regulate androgen receptor activity through changes to the acetylation status of the receptor. *J Biol Chem*. 2002;277(29):25904-25913.
43. Shang YF, Myers M, Brown M. Formation of the androgen receptor transcription complex. *Mol Cell*. 2002;9(3):601-610.
44. Casati L, Sendra R, Sibilia V, et al. Endocrine disrupters: the new players able to affect the epigenome. *Front Cell Dev Biol*. 2015;3:37.