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Lactational Exposure to Di-(2-Ethylhexyl) Phthalate (DEHP) Alters GnRH Expression in Hypothalamus of Male and Female Pubertal Albino Wistar Rats

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ABSTRACT

Di-(2-ethylhexyl) phthalate (DEHP) is a ubiquitous environmental contaminant, an established reproductive and developmental toxicant. Amid concerns that chronic low-dose exposures to endocrine disrupting chemicals (EDCs) may be contributing to a decline in fertility in humans, recent interest has turned to elucidating how reproductive neuroendocrine systems may be perturbed by EDC exposures during early critical life stages. In this investigation, effects of lactational exposure to DEHP on the hypothalamus of male and female albino pubertal rats were studied. Mother rats received phthalate (DEHP) through oral gavage (Vehicle - Olive oil); Group I: Control, Group II: 1 mg DEHP/ Kg.B.Wt./day, Group III: 10 mg DEHP/Kg.B.Wt./day and Group IV: 100 mg DEHP/Kg.B.Wt./day. The treatment periods were from PP-1 to PP-21 (post-partum). The male and female offspring rats were sacrificed on PND 60. The results showed that DEHP has a differential effect on male and female rats. IHC results showed that DEHP significantly decreased GnRH neurons in male rats whereas in female rats there was an increase in GnRH neurons. The mRNA expression of GnRH1, Kisspeptin1, Androgen Receptor and Aromatase were decreased in male rats whereas increased in female rats. Therefore, the present study shows that early postnatal exposure to DEHP effects neuroendocrine control of reproduction.

Keywords: Phthalates, Hypothalamus, Gonadotropin releasing hormone 1, Kisspeptin 1, Aromatase, Androgen receptor

Abbreviations: DEHP: Di-(2-Ethylhexyl) Phthalate; EDC: Endocrine Disrupting Chemicals; GnRH: Gonadotropin Releasing Hormone; AR: Androgen Receptor; PP: Post-Partum; PND: Postnatal Day, DG: Dentate Gyrus; CA: Cornus Ammonis; SO: Stratum Oriens; POA: Preoptic Area; IHC: Immunohistochemical Localization; PCB: Polychlorinated Bi-Phenyl; GPR54: G-Protein Coupled Receptor-54

INTRODUCTION

Phthalates are a family of chemicals used in many consumer products, including building materials, cosmetics, clothing, pharmaceuticals, medical devices, toys, food packaging, cleaning materials and insecticides [1]. Annually, more than three million metric tons of phthalates are produced globally [2]. Because of their widespread use, human population, domestic animals, and wildlife are regularly exposed to phthalates. People may be exposed in the work environments [3]. In addition, several biomonitoring studies have revealed a widespread exposure of the non-occupational human population [4] via food from plastic containers and via inhalation of dust in domestic environments. Of the different phthalate compounds, di-2-ethylhexyl phthalate (DEHP) is produced in extremely large quantities. DEHP is a ubiquitous environmental contaminant, an established reproductive and developmental toxicant [5]. Considering that DEHP readily crosses the placenta and accumulates in the fetus [6] it could potentially act either directly on the fetus and/or indirectly on the placental barrier, resulting in its teratogenic effects.

Exposure to environmental endocrine disrupting chemicals (EDCs) like phthalates, during critical developmental periods, particularly gestation and infancy, are consistently linked to impairments in homeostatic, endocrine and neurobiological processes in adulthood [7]. Amid concerns

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that chronic low-dose exposures to EDCs may be contributing to a decline in fertility in humans [8] recent interest has turned to elucidating how reproductive neuroendocrine systems may be perturbed by EDC exposures during early critical life stages. As the hypothalamic control of reproduction develops in a sexually dimorphic manner due to sex differences in gonadal steroid hormone actions in the brain, it is plausible to hypothesize that some of the links between perinatal EDCs and the diminution in reproductive competency may be due at least in part to reprogramming of the neonatal hypothalamus by these compounds.

Administration of DEHP to dams has been demonstrated to produce adverse neurobehavior in mice offspring [9]. Several neurodegenerative areas in rat brain have also been identified after DEHP exposure [10]. *In utero* and lactational DEHP exposure inhibited sexually dimorphic central nervous system (CNS) development Moore et al. [11] and Andrade et al. [12] have shown that DEHP alters the activity of aromatase in young rats following perinatal exposure. This activity is crucial for masculinization of the brain. In utero exposure to DEHP alters the lipid metabolome in the fetal brain, which may lead to aberrant neurodevelopment. Xu et al. [13] and Smith et al. [14] have shown that exposure to di(2-ethylhexyl) phthalate (DEHP; 10 mg/kg, i.p.) from p16 to p22 reduced axonal markers in the CA3 distal stratum oriens (SO) and reduced cell density of both immature and mature neurons in the dentate gyrus (DG) and CA3, respectively, of hippocampus in male rats.

The hypothalamus, located at the base of the brain, is the site of the neurons that controls central neuroendocrine function in vertebrates. The neuroendocrine hypothalamus, which serves as a major interface between the central nervous system and the rest of the body, signals to the periphery through the release of hypothalamic releasing/inhibiting hormones into the portal capillary system that leads to the anterior pituitary gland [15]. GnRH is a decapeptide released into the hypothalamic portal circulation, through which it stimulates the synthesis and release of LH and FSH from the anterior pituitary gland. The pulsatile release of GnRH is essential for sexual maturation and maintenance of the ovulatory cycle [16]. Kisspeptins are peptide products of the KiSS-1 gene, which was first discovered by Lee et al. [17] as a metastasis suppressing gene in malignant melanoma cells. Although kisspeptins and gpr-54 were first described in relation to cancer metastasis, they were subsequently shown to play a pivotal role in the control of the hypothalamic-pituitary-gonadal (HPG) axis via regulation of gonadotrophin-releasing hormone (GnRH) secretion [18,19]. The actions of androgens within target cells are transduced by the low abundance intracellular AR, the number 4 member of the NR3C subgroup of a nuclear receptor superfamily that mediates the action of steroid hormones. Aromatase plays an essential role in sexual differentiation of the brain in many mammalian species [20]. Aromatase (CYP19) is considered to be one of the potential EDC targets because modulation of its expression and function can dramatically alter the rate of estrogen production, disturbing the local and systemic levels of estrogen and thus may lead to disruption of estrogen-related biological processes.

The effect of lactational exposure of DEHP on neuroendocrine control of reproduction has not been studied so far. The aim of this study was to investigate how postnatal exposure to DEHP effects neuroendocrine control of reproduction focusing on GnRH neurons and the possible mechanism behind it in pubertal male and female albino rats.

MATERIALS AND METHODS

Chemicals

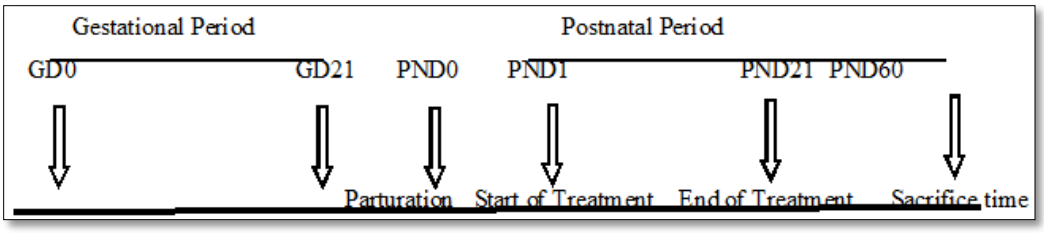
Di-2-ethyl hexyl phthalate (DEHP), TRI Reagent, Chloroform, Isopropanol and all other chemicals of molecular grade were purchased from M/S Sigma-Aldrich Pvt. Limited (USA).

Animals

Healthy adult pregnant female albino rats of Wistar strain (*Rattus norvegicus*) weighing about 180-200 g (100 days) were used in the present study. The study protocol was reviewed and approved by the institutional ethical committee (Ref No. IAEC No: 01/01/10). The animals were housed in clean polypropylene cages, maintained in air-conditioned animal house with constant photoperiod of 12 h light/dark cycle. The animals were fed with pellet diet (Gold Mohur Ltd., Mumbai, India) and drinking water ad libitum.

The treatment procedure

Pregnant rats were divided into four groups, each consisting of six animals; Group I: Control, Group II: 1 mg DEHP/Kg.B.Wt./day, Group III: 10 mg DEHP/Kg.B.Wt./day and Group IV: 100 mg DEHP/Kg.B.Wt./day. The number of litters were culled down to six each mother with sex ratio 3:3. From PND (Post natal day)-1 to PND-21 mother rats received phthalate (DEHP) through oral gavage (Vehicle-Olive oil). The pubertal male and female rats were sacrificed on PND 60. All the female rats were killed in diestrous phase. During and after the treatment period the animals were monitored for morphological changes such as body weight, feed and water consumption **(Flowchart 1)**.



Flowchart 1. Treatment schedule.

Mother rats were treated with DEHP from PND1-21. After weaning at PND 23 pups were housed separately by sex per cage until termination of experiment (PND 60).

Sample collection and preparation

The animals were sacrificed, the brain was excised immediately and washed in ice-cold physiological saline repeatedly; hypothalamus was separated and weighed accurately. 100 mg fresh tissue was weighed and processed for RNA isolation.

RNA ISOLATION AND REAL TIME PCR

The total RNA was isolated by using TRI Reagent (Sigma) [21]. The concentration and purity of total RNA were determined by absorbance at 260/280 nm in a UV spectrophotometer. If the ratio of A260/280 is 1.8-2.0, then 1.5 µg of total RNA was used to synthesize Complementary DNA using a cDNA synthesis kit (IScript, Bio-Rad, USA). Real time-PCR was carried out in MX3000p PCR system (Stratagene, Europe). Reaction was performed using KAPA-SYBR green fast PCR master mix PCR kit (It contains all the PCR components along with SYBR green dye). The data were normalized by comparing threshold cycle ratios between the candidate genes and a housekeeping gene 18S rRNA. The data were analyzed by the comparative CT method [22]. The primer sequences used are listed in **Table 1**.

Table 1. List of primer sequences used for RT-PCR.

Gene	Forward Primer	Reverse primer	Gene Bank Accession No.
GNRH 1	AGCCAGCACTGGTCCTATGGGT	TCAGGCAGTGTACCTGCTCGCT	NM_012767.2
Kisspeptin 1	GGCAAAAGGGCCCGCGGTAT	GATCAGGCGACTGCGGGTGG	NM_181692.1
Androgen Receptor	GGTAATATCCGAAGGCAGCA	TCCCCTGGACTCAGATGTTC	NM_012502.1
Aromatase (Cyp 19)	AGGAGCCTTTACCTGCTCTTGGT	GCCCTTGAGTGGGTAGAGTGACG	NM_017085.2

IMMUNOHISTOCHEMISTRY

Rats were anaesthetized and perfused transcardially with PBS (0.1 M, pH 7.4) to flush the blood from the vascular system, followed by 4% paraformaldehyde in PBS. Following perfusion brains were carefully removed and stored in the perfusion fixative for 2-3 days at 4°C. The brains were coronally sectioned at 10 µm thickness using a Rotary microtome. A profile of 10 sections was processed simultaneously from control and DEHP treated groups to ensure uniformity of immunostaining. Sections were washed twice in xylene (to dewax) for 15 min each followed by Absolute alcohol and graded alcohol wash. The sections were heated in tri-sodium citrate at 95°C for 10 min, cooled and endogenous peroxidase was quenched by adding 3% H_2O_2 in methanol for 10 m in dark. Sections were washed and blocked for 30 min in 1.5% blocking buffer followed by overnight incubation with mouse anti-GnRH monoclonal antibody (Santa-Cruz, USA) (1:200). After three washes with TBS-T for 5 min each the sections were incubated in anti-mouse biotin conjugated secondary antibody (1:500) (Santa-Cruz, USA) for 30 min. After washing with TBS-T the sections were incubated in Avidin Biotin complex (Vectastein ABC kit, Elite pk-6100) for 30 min, rinsed in buffer and developed in 3,3-diaminobenzidine/peroxidase (Vector Laboratories, USA) reaction. The sections were counterstained with haematoxylin for 5 s, cover slipped with DPX and photographed with Nikon Microscope Eclipse 80i (Japan).

QUANTITATIVE ANALYSIS

Quantification of the GnRH positive neurons was carried out with a reticule fitted in a Nikon Microscope Eclipse 80i (Japan). The POA area was localized with the help Brain maps is focused in 40X magnification and counted for all the GnRH positive neurons lying within the reticule. The data

were statistically analyzed by means of a one-way ANOVA followed by SNK's test and expressed as GnRH neurons per field coming under a reticule.

STATISTICAL ANALYSIS

Data were statistically analyzed by One-way analysis of variance (ANOVA) followed by Student's – Newman Keul's test (SNK) to assess the significance of individual variations between the treatment groups using SPSS (version 20). p value <0.05 was considered as statistically significant.

RESULTS

Body weight and hypothalamus weight

The body weight of 1 and 10 mg DEHP treated animals did not show any significant difference compared to control animals on PND 60 whereas the total body weight of 100 mg DEHP treated group was significantly decreased compared to control group in both male and female rats **(Figures 1A and 1B)**. Hypothalamus weight was significantly decreased in 100 mg DEHP treated animals compared to control group whereas 10 and 100mg DEHP treatment did not show any significant change in hypothalamus weight **(Figures 1C and 1D)**.

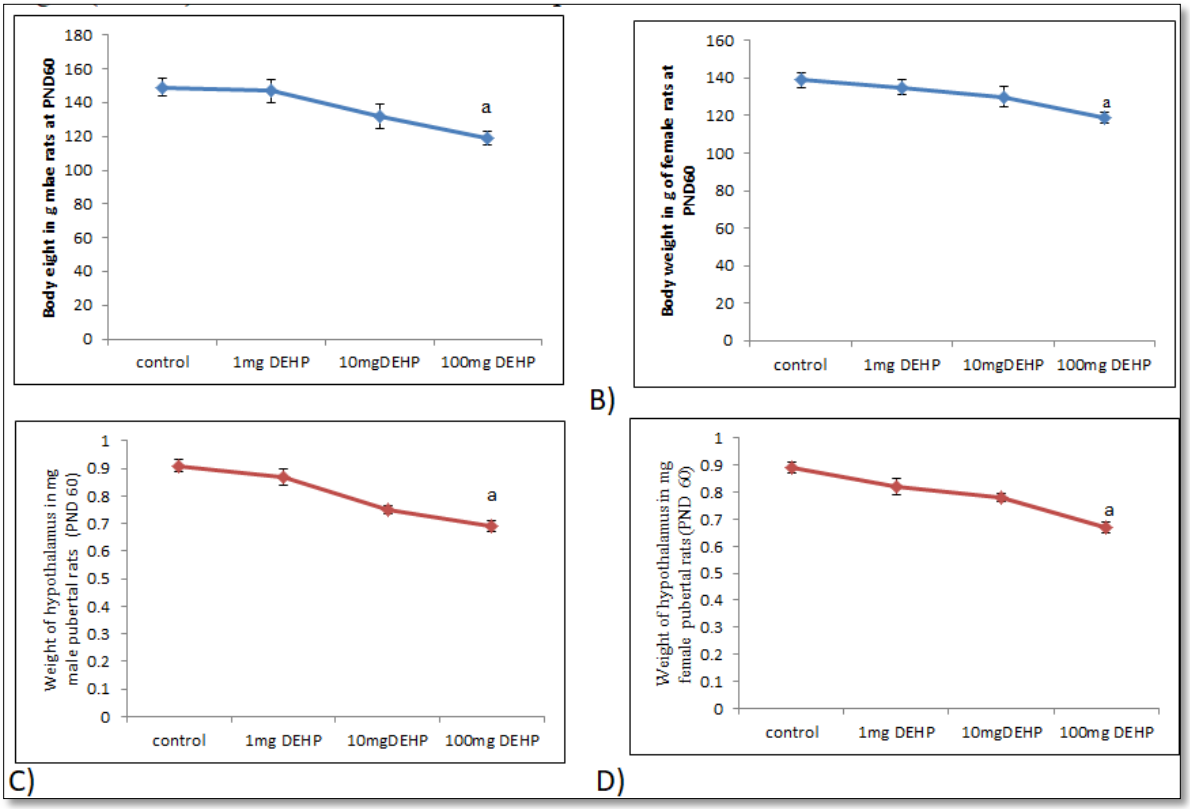


Figure 1. Effect of lactational exposure to DEHP on body weight (A & B) and hypothalamus weight (C & D) of male and female albino pubertal rats.
Each bar represents the mean ± SEM of six animals. The data was analyzed by one way ANOVA followed by Student's–Newman–Keul's test using SPSS 17.5 version. "a" Represents statistical significance between control versus DEHP treatment groups at P<0.05

GnRH expression

GnRH release drives reproduction throughout the life cycle, and this is the primary stimulus to the rest of the reproductive axis. Lactational exposure of DEHP down-regulated the mRNA expression of GnRH in the hypothalamus of male pubertal Wistar rats in 10 and 100 treated groups **(Figure 2)**. The mRNA expression of GnRH was up regulated in 10 mg and 100 mg treated groups when compared to that of control group in female rats. However, 1 mg DEHP treated group didn't show any significant change in GnRH expression.

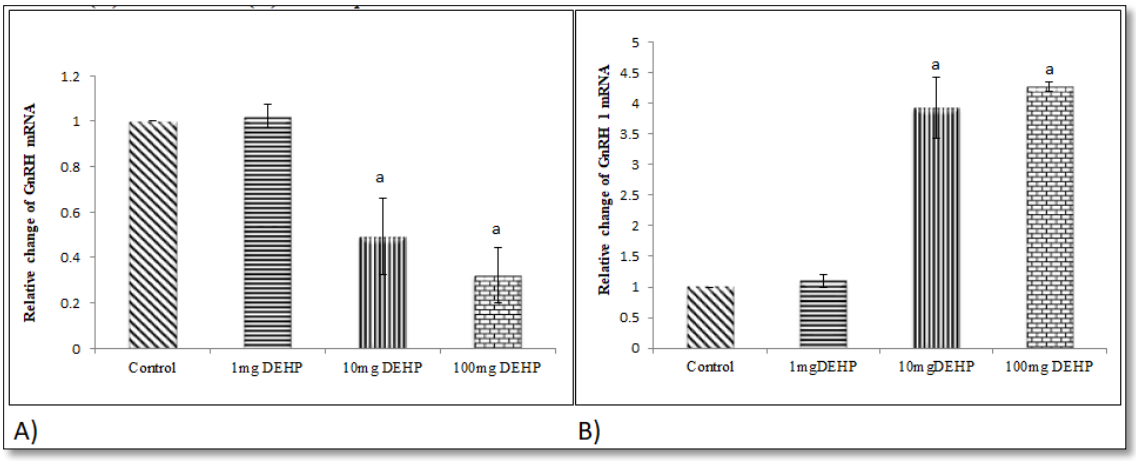


Figure 2. Effect of lactational exposure to DEHP on mRNA expression of GnRH 1 in the hypothalamus of male (A) and female (B) albino pubertal rats.
The data was analyzed by the comparative C_T method. Each bar represents the mean ± SEM of three independent observations. "[a]" Represents statistical significance between control versus DEHP treatment groups at P<0.05 level using Student's–Newman–Keuls test

Kisspeptin 1 expression

Kisspeptin expression has been identified in hypothalamic nuclei important in the regulation of GnRH secretion. Kisspeptins are potent secretagogues for GnRH and the Kiss1 gene is a target for regulation by gonadal steroids (e.g. estradiol and testosterone), metabolic factors (e.g. leptin), photoperiod and season. **Figure 3** shows the effects of lactational exposure of DEHP on mRNA expression of the kisspeptin 1 in the hypothalamus of pubertal Wistar male and female rats. The mRNA expression of Kisspeptin 1 was down-regulated in 10 mg and 100 mg DEHP treated groups when compared to that of control group, whereas up regulated in female rats. However no significant change was observed in 1 mg treated group.

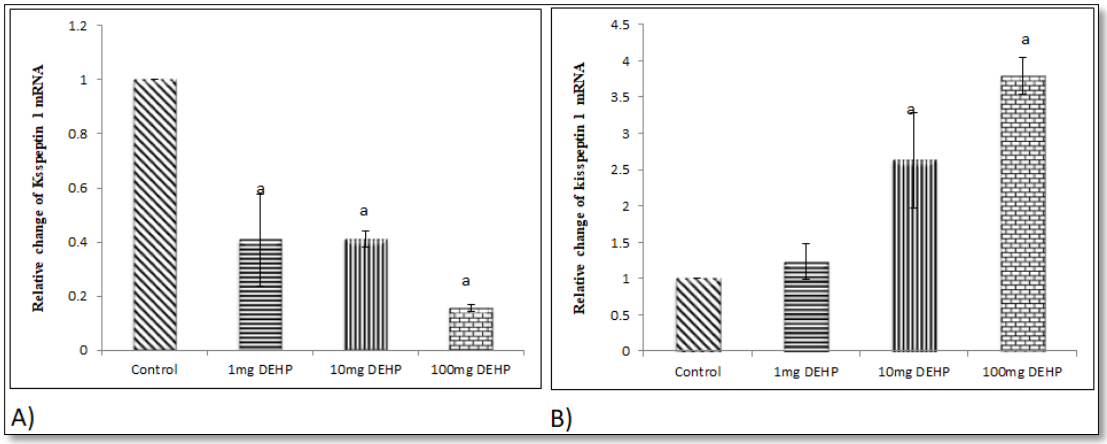


Figure 3. Effect of lactational exposure to DEHP on mRNA expression of kisspeptin 1 in hypothalamus of male (A) and female (B) albino pubertal rats.
The data was analyzed by the comparative CT method. Each bar represents the mean ± SEM of three independent observations. "[a]" Represents statistical significance between control versus DEHP treatment groups at P<0.05 level using Student's–Newman–Keuls test

Androgen receptor (AR) expression

The actions of androgens within target cells are transduced by the low abundance intracellular AR, the number 4 member of the NR3C subgroup of a nuclear receptor superfamily that mediates the action of steroid hormones. **Figure 4** shows the effects of lactational exposure of DEHP on mRNA expression of the Androgen Receptor (AR) in the hypothalamus of pubertal Wistar male and female rats. The mRNA expression of AR was down-regulated in 10 mg and 100 mg treated groups when compared to that of control group and up regulated in female rats. However, no significant change was observed in 1mg treated group.

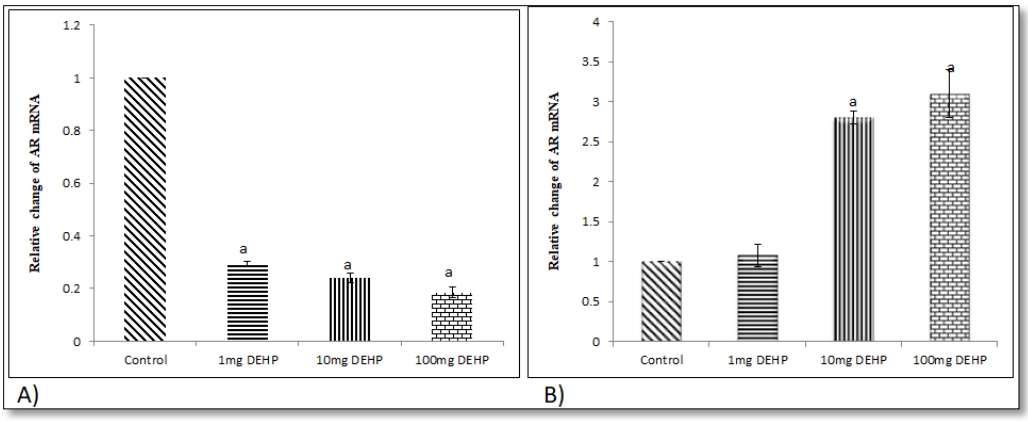


Figure 4. Effect of lactational exposure to DEHP on mRNA expression of androgen receptor (AR) in the hypothalamus of male (A) and female (B) albino pubertal rats.
The data was analyzed by the comparative C_T method. Each bar represents the mean ± SEM of three independent observations. "a" Represents statistical significance between control versus DEHP treatment groups at P<0.05 level using Student's–Newman–Keuls test

Aromatase expression

Aromatase converts circulating androgens to active estrogenic metabolites in specific neural target tissues or may serve as part of endogenous neurosteroid machinery that supplies estrogen directly to specific regions of the brain. **Figure 5** shows the effects of lactational exposure of DEHP on mRNA expression of the aromatase in the hypothalamus of pubertal Wistar male and female rats. The mRNA expression of aromatase was down-regulated in 10 mg and 100 mg treated groups when compared to that of control group and up regulated in female rats. However, no significant change was observed in 1 mg treated group.

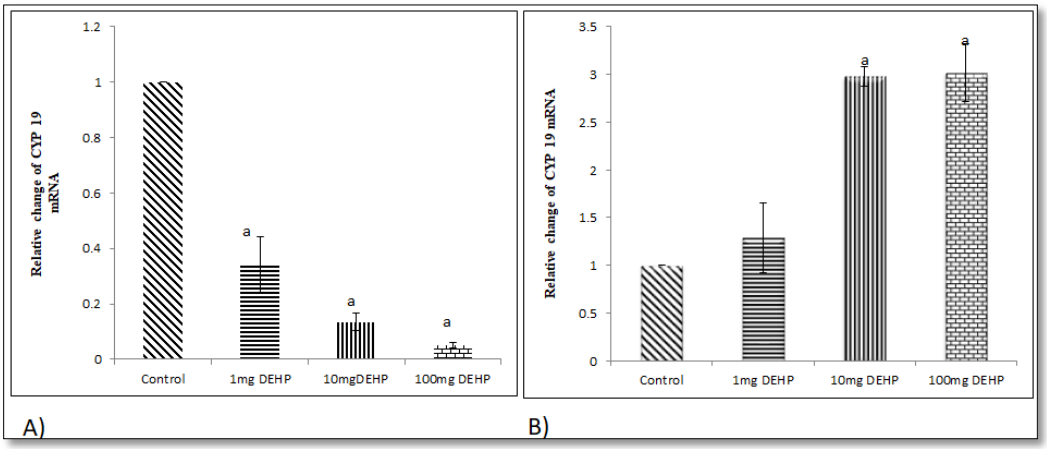


Figure 5. Effect of lactational exposure to DEHP on mRNA expression of Aromatase (cyp 19) in the hypothalamus of male (A) and female (B) pubertal Wistar rats.
The data was analyzed by the comparative C_T method. Each bar represents the mean ± SEM of three independent observations. "a" Represents statistical significance between control versus DEHP treatment groups at P<0.05 level using Student's–Newman–Keuls test

Effect of lactational exposure to DEHP on immunohistochemical localization of GnRH neurons in the POA of hypothalamus

A representative low power (40X) micrograph showing immunohistochemistry of GnRH (dark brown) immunoreactive cells, at the level of the POA is shown in **Figure H**. Higher power micrographs (100X) of labeled cells are shown in **Figure 1**, **I and J**, respectively. The number of GnRH positive nuclei were counted using reticule fitted in a Nikon Microscope Eclipse 80i (Japan) and compared between vehicle control (olive oil) and DEHP 1, 10 and 100 mg treated groups. DEHP treatment caused a significant increase in the number of cells that expressed GnRH in female pubertal rats whereas there was a significant decrease in GnRH positive cells in males at higher doses (10 and 100 mg DEHP) **(Figure 2)**.

DISCUSSION

When neuroendocrine homeostasis is disrupted by environmental endocrine-disrupting chemicals, a variety of perturbations can ensue, particularly during critical developmental time periods. There is increasing evidence that the central neuroendocrine systems are targets of endocrine-disrupting chemicals (EDCs) [15]. The timing of exposure to an EDC is crucial in determining its ultimate effect. It is recognized that there are critical developmental periods during which neuroendocrine systems are modulated by steroid and other hormones. For example, early life exposure to endogenous androgens or estrogens, particularly in fetal life and infancy, organizes the brain in a sexually dimorphic manner (i.e., resulting in morphological and functional differences between males and females) that becomes activated later in life [23].

Exposure to exogenous substances such as EDCs is likely to have more profound detrimental consequences in developing organisms than in adults [24]. This concept is now referred to as the "fetal/developmental basis of adult disease" and is highly applicable to neuroendocrine systems. For example, in the case of the HPG axis, early life exposures to environmental EDCs can permanently alter sexual development, resulting in females that are masculinized or defeminized and males that are feminized or demasculinized [25,26]. These effects of EDCs on brain sexual differentiation are manifested as changes in reproductive development and may be detrimental to fertility and reproductive success. Therefore, the fetal/developmental basis of adult disease is a critical concept for neuroendocrine disruption **(Figure 6)**.

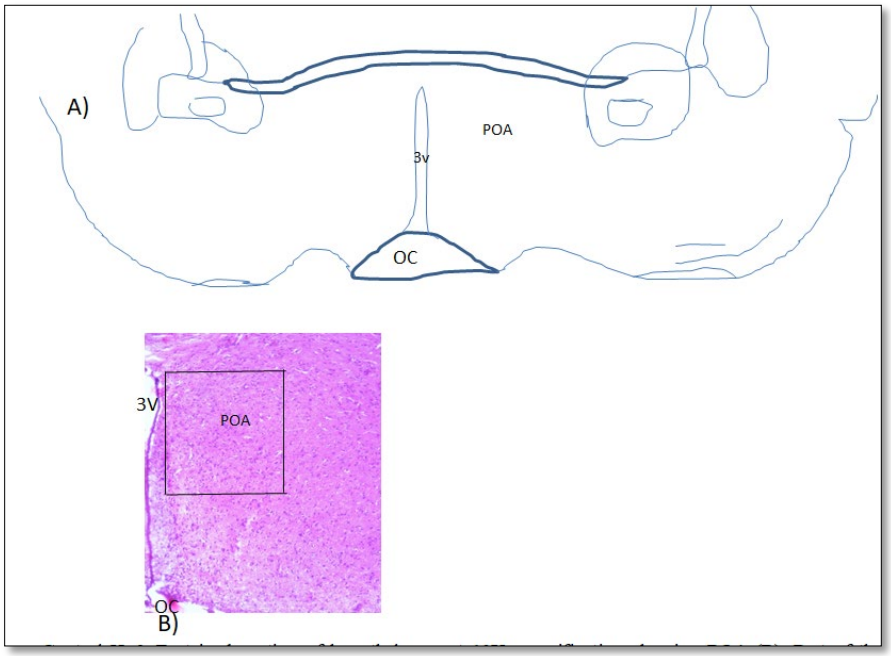


Figure 6. Control Haematoxylin and Eosin (H & E) stained coronal section of hypothalamus. Graphical representation of frontal hypothalamus showing the localization of POA (Preoptic area) (A).
Control H&E stained section of hypothalamus at 10X magnification showing POA (B). Part of the third ventricle is shown. 3V-third ventricle, OC-optic chiasma

The hypothalamic GnRH neurons control reproductive function in all vertebrates [27]. The data on localization of GnRH neurons at the level of POA in hypothalamus shows that at higher dosage DEHP significantly decreased the number of GnRH positive neurons in male pubertal rats **(Figure 7)** while as in female rats there was an increase in GnRH positive neurons at higher dosage groups, suggesting that DEHP has differential effects on male and female rats **(Figure 8)**. The IHC data was well supported by qPCR data where we observed that we found that DEHP caused a dose dependent decrease in GnRH mRNA in pubertal male rats with 100 mg treated group showing the maximum effect. In female pubertal rats the trend was opposite with 10 and 100 mg DEHP groups showing a significant increased GnRH mRNA expression. There is *in vitro* and *in vivo* evidence that GnRH neurons can be direct targets of EDCs. We propose that DEHP may act in a similar manner as PCBs since both have been shown to act via aryl hydrocarbon receptor and both are endocrine disruptors. When GT1-7 cells were treated with a PCB mixture, Aroclor 1221 or Aroclor 1254, these cells had elevated GnRH gene expression from low dose treatment and there was relatively little effect by higher doses [27]. In that same study, there was a stimulation of GnRH peptide release into the medium

by Aroclor 1221 but not Aroclor 1254. Further, a nuclear estrogen receptor antagonist, ICI 182,780, blocked some of these effects of PCBs, suggesting a mechanism that is partially mediated by this receptor. Dickerson et al. [28] has shown those PCBs (PCB 74, 118 or 153) or a mixture of the three increased GnRH peptide levels at low doses while as decreased GnRH peptide levels at higher dosage levels.

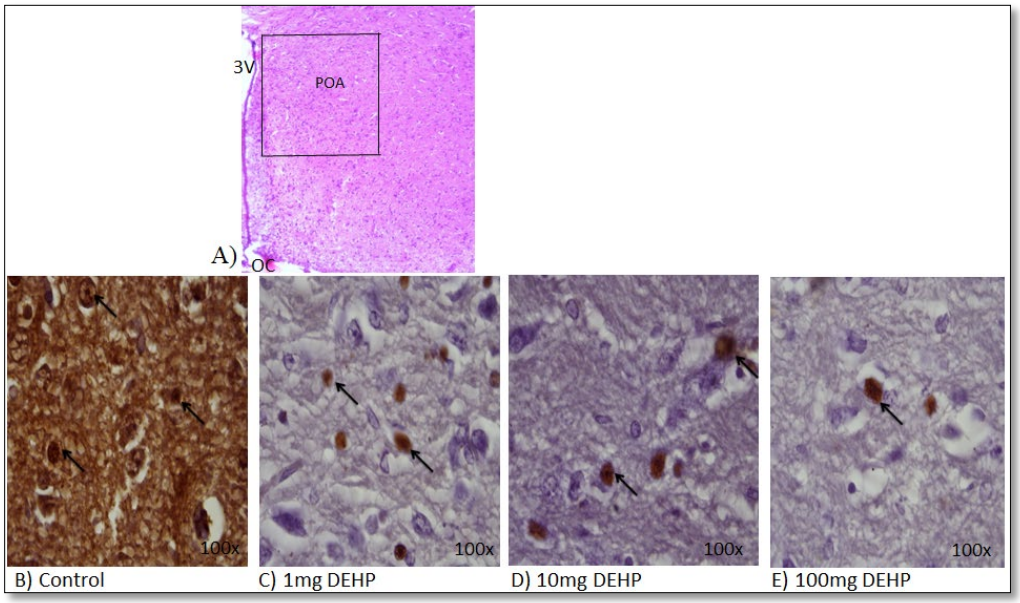


Figure 7. DAB immunohistochemistry for GnRH in pubertal male rats. Low magnification representation of control rat hypothalamus at 10X (A), the area inside the box represents the area shown at higher magnification and high magnification (B-E; 100X) in the POA.
B-E Representative micrographs of GnRH positive nuclei (dark brown) in the POA that were detected using the DAB/peroxidase reaction.Black arrows indicate GnRH nuclei

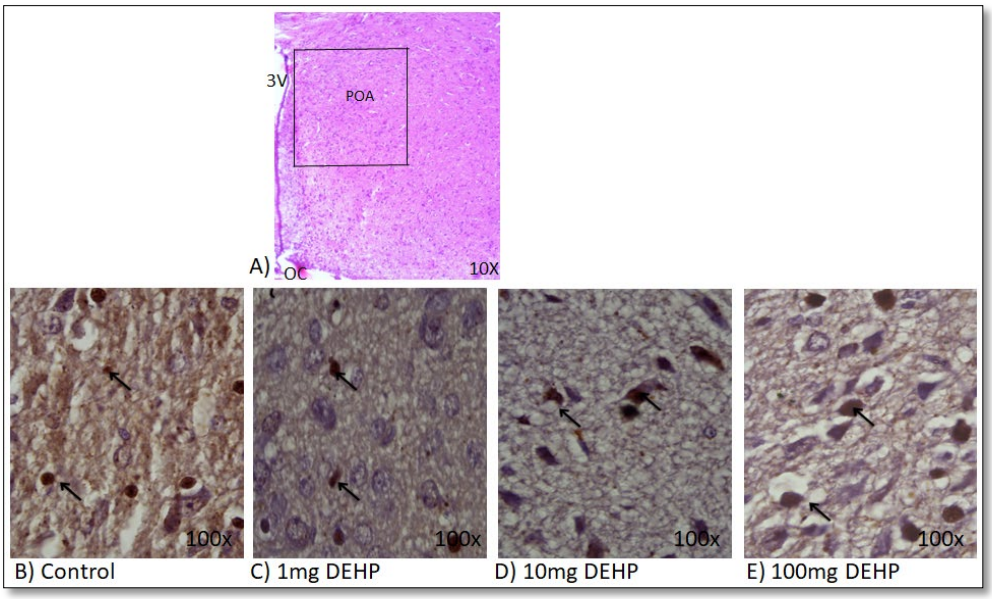


Figure 8. DAB immunohistochemistry for GnRH in pubertal female rats. Low magnification representation of control rat hypothalamus at 10X (A), the area inside the box represents the area shown at higher magnification and high magnification (B-E; 100X) in the POA.
B-E GnRH positive nuclei (dark brown) in the POA that were detected using the DAB/peroxidase reaction at 100X. Black arrows indicating GnRH positive neurons

To further understand how DEHP altered GnRH, Kisspeptin 1 mRNA expression was studied and it was found to be decreased in male and up regulated in female rats in the same manner as that of GnRH. Kisspeptins increase gonadotropin levels by stimulating GnRH secretion [29]. Evidences suggest that kisspeptins increase gonadotropin secretion by stimulating GnRH secretion. First, pretreatment with the GnRH antagonist sacyline [30] or cetrorelix [31], prevents kisspeptin-induced gonadotropin secretion. Second, ICV administration of kisspeptin increases GnRH secretion (as measured in cerebrospinal fluid) concurrent with LH secretion in female sheep [32]. Kisspeptin appears to stimulate gonadotropin release by acting on GPR54 alone, because kisspeptin does not elicit gonadotropin release in the GPR54 KO mouse [19]. Therefore, we propose that DEHP may have altered GnRH levels both directly and indirectly via kisspeptin 1. Navarro et al. [33] have shown that maternal exposure to a complex mixture of chemicals/EDCs, at environmental concentrations, affects the fetal kisspeptin/GPR54 neuroendocrine system **(Figure 9)**.

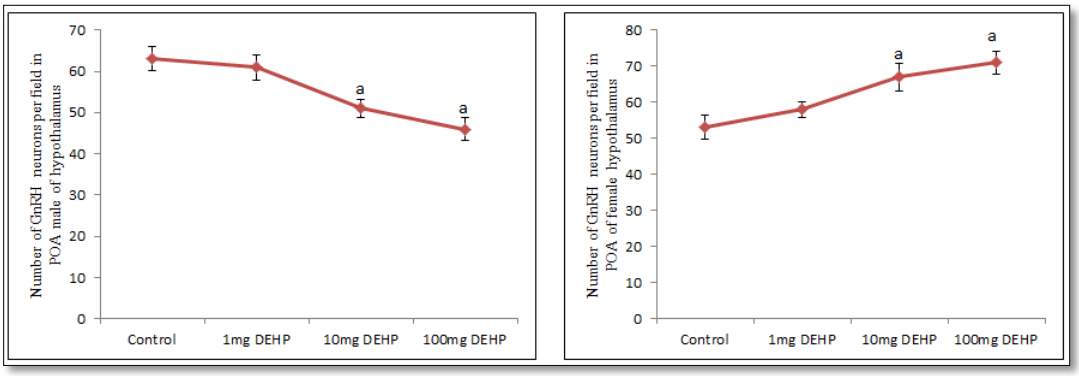


Figure 9. Quantification of GnRH neurons in POA of hypothalamus. Each bar represents mean ± SEM of three observations. *The data was statistically analyzed by one way ANOVA followed by Student's–Newman–Keul's test using SPSS 17.5 version. "a" Represents statistical significance between control versus DEHP treatment groups at P<0.05*

It has been shown that in hypothalamus, androgens regulate aromatase mRNA via androgen receptor-mediated transcription [34]. DEHP caused a dose dependent decrease in AR in male rats and an increase in AR mRNA at all doses in the present study. Butyl benzyl phthalate (BBP) was shown to bind AR *in vitro* [35]. Activation of AR is critical for both defeminization and masculinization of the developing brain and body [36]. In addition, extensive co-localization of androgen receptor and aromatase was observed in the neuroendocrine brain [37] and a potential androgen responsive element and SF-1 site is present at the 5`-end of the brain specific exon lf of aromatase [38]. ARs may regulate aromatase expression or activity to affect estrogen receptor activation or may act independently of estrogen receptors to influence hypothalamus morphology. In the present study, lactational exposure to DEHP decreased aromatase mRNA in male rats and increased aromatase mRNA expression in a dose dependent manner following the same trend as that of AR mRNA. Since AR has been reported to regulate aromatase transcription, the observed changes in aromatase mRNA expression may have been accounted by AR. The current study suggests that early postnatal exposure to DEHP may have detrimental effects on developing organisms. The episodic modes of GnRH secretion from the hypothalamus and of GnRH receptor (GnRHR) activation in pituitary gonadotrophs are essential for optimal gonadotropin synthesis and secretion and ultimately for normal reproductive function.

Therefore, it is suggested that early postnatal exposure to DEHP may affect neuroendocrine aspects of reproduction and decrease the reproductive ability of the developing organism by causing early puberty or delayed puberty. It is clear that further work is necessary to understand the underlying mechanisms of these EDCs on targets in the hypothalamus.

CONCLUSION

DEHP (10 and 100 mg doses) altered the mRNA expression of the genes studied in the present study. Therefore, it is concluded from the present study that early postnatal exposure to DEHP may have effects upon the developing organism reproductive ability by not only affecting gonads but by affecting neuroendocrine control of the reproduction thereby having long lasting effects on the developing organism.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

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