Journal of Endocrinology and Reproduction, *Vol 20(2), 92-101, 2016, DOI: 10.18311/jer/2016/18116*
ISSN (Print) : 0971-913X
ISSN (Online) : 2455–1244

Gestational Exposure to Di(2-ethylhexyl)phthalate Modifies the Expression Pattern of Genes Controlling Thyroid Hormone Biosynthesis in Puberal Rat Progeny

Sambavi Elangovan , Aruldhas M. M.*, Suganya S., Rajesh P, Suthagar E., Navin A. K., Shobana N., Ravi Sankar B. and Ilangovan R.

Department of Endocrinology, Dr ALM PG Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai – 600113, Tamil Nadu, India; aruldhasmm@gmail.com

Abstract

Di(2-ethylhexyl) phthalate (DEHP), a plasticizer, is known to disrupt thyroid functions but the underlying molecular mechanism remains obscure. The present study was conducted testing the hypothesis that gestational exposure to DEHP would modify the expression of specific genes controlling biosynthesis and action of thyroid hormones in the male progeny at puberal age. Pregnant rats were administered with DEHP [1, 10 and 100 mg (in olive oil)/Kg b.wt./day] from embryonic day 9 to 21 through oral route. The pups were sacrificed on post-natal day 60. Enzyme Immuno-Assay (EIA) revealed a dose-dependent decrease in serum 3,5,3' triiodothyronine (T_3) and L-thyroxine (T_4) titres in DEHP-treated rats. Real-time RT-PCR and western blot analyses of thyroidal genes revealed decreased expression level of sodium/iodide symporter (*Nis)* and thyroid hormone receptor α (*Trα)*, whereas the expression of thyroid stimulating hormone receptor (*Tshr*), thyroid hormone receptor β (*Trβ*) and pendrin *(Pds)* increased. While western blot detection showed decreased expression level of thyroperoxidase *(Tpo),* RTPCR data pointed out augmented expression. Western blot detection of transcriptional factors showed decreased expression levels of fork-headbox e1 (Foxe1) and hematopoietically expressed homeobox (Hhex), whereas thyroid transcription factor-1 (Ttf-1) and paired-box domain 8 (Pax8) increased. Our study demonstrates, for the first time, that gestational exposure to DEHP affects the expression of genes controlling thyroid hormone synthesis in puberal rat progeny, and the hypothyroid state in these rats may be linked to decreased expression of *Nis, Tpo*, Foxe1 and Hhex.

Keywords: Pendrin, Sodium/Iodide Symporter, Thyroperoxidase, Thyrotrophin Receptor, Hematopoietically Expressed Homeobox

1. Introduction

Phthalates and phthalate esters are man-made chemicals, mainly used to impart flexibility, pliability and elasticity to plastics and are, therefore, known as "plasticizers"[1]. These chemicals enter the environment *via* various products including medical tubing and blood bags, food handling and storage, electrical devices and toys, and also non-PVC products such as paints, cosmetics and lacquers, which contain the chemical[1,2]. Phthalates are structurally non-covalently bound in products and can, therefore, easily leach out to contaminate a variety of household and industrial products[3] due to their persistent presence in the environment, domestic animals, wildlife and humans; the susceptible population like pregnant women and children are potentially exposed to these compounds continuously through oral, nasal and dermal routes[4]. Phthalates and their metabolites have been found in several biological fluids including urine, serum, breast milk, saliva and amniotic fluid in humans[5,6]. DEHP is known to disrupt

*Author for correspondence

endocrine functions[7,8]. Recent reports from our laboratory indicated disruption of testicular histoarchitecture and repression of critical genes in Leydig cells of adult rats with gestational exposure (ED 9-21) to different doses of DEHP[9] and impairment of pancreatic β-cell functions[10].

Disruption of thyroid gland functions by DEHP is known[7]. Long-term (13 weeks) dietary exposure of DEHP led to reduced thyroid follicle size and colloid density in male and female Sprague-Dawley rats[11]. On the contrary, short-term (3 week) exposure to DEHP caused increase in the number and size of lysosomes, enlargement of Golgi apparatus and damage to mitochondria of thyroid gland in rats[12]. Wistar rats fed DEHP along with the hypolipidemic drugs clofibrate and fenofibrate (50 to 1000 mg/kg/day) through oral route for 12 weeks exhibited shrunken colloid accompanied by calcium-rich inclusions[13,14]. Another study treated Wistar rats with DEHP (50-2000 mg/kg) and a related compound di-octyl phthalate (DOP, 2 g/kg) for 3 days to 9 months and showed decreased colloid density and increased rate of thyroglobulin turnover[15]. Hinton et al., reported a non-significant time-and dose-dependent increase in serum T_3 and a time-dependent decrease in T_4 in male Wistar rats. Rat thyroid FRTL-5 cell line challenged with non-cytotoxic doses of DEHP (100 μM to 1 mM) showed enhanced uptake of iodide[16]. Breous et al.,[17] reported that DEHP-induced increase in iodide uptake in rat thyroid cell line (PCC13) was the consequence of augmented transcription of *Nis*.

Epidemiological studies found an inverse association between urinary Mono-2-Ethylhexyl Phthalate (MEHP) concentration and free T_4 and total T_3 levels, but not with Thyroid Stimulating Hormone (TSH) after adjusting for other covariates; Mono-2-Ethyl-5-Hydroxyhexyl Phthalate (MEHHP) concentration was positively associated with FT_4, but not with TSH and T_3, in a subgroup of 208/408 men[18]. Meeker and Ferguson[19] conducted a study in 1,346 adults (age≥20 years) and 329 adolescents (aged 12–19 years) and reported an inverse relationship between urinary metabolites of DEHP and total and free T_4, total T_3 and Tg, and a positive association with TSH. Although the above findings indicated that DEHP and its metabolites can disrupt thyroid hormone homeostasis, the underlying molecular mechanism remains elusive. Thus, the existing literature suggests that phthalates are potent thyroid disrupting chemicals, but the information is confined to adult animals and humans only. It is not known if embryonic exposure to DEHP can disrupt thyroid function during postnatal period. The imprinting effect of embryonic exposure to EDCs on various organ systems during postnatal life is known[20]. Therefore, it is imperative to understand the impact of embryonic exposure to phthalates on thyroid function during post-natal life.

Based on the above literature background, it was hypothesized that gestational exposure to DEHP may modify the expression of specific genes controlling biosynthesis and action of thyroid hormones in the progeny at puberal age. The hypothesis was tested in male Wistar rats by detecting the expression of *Tpo*, *Nis*, *Pds*, and *Tshr* that control thyroid hormone synthesis. Additionally, *Trα* and *Trβ*, TTF-1, FOXE1, PAX 8 and HHEX that control the expression of *Tshr, Nis, Tg*, *Tpo* and *Pds*[20,21] were studied in puberal rats exposed to different doses of DEHP during gestational period.

2. Materials and Methods

2.1 Animals, Doses and Treatment

The experiments in Wistar rat (*Rattus norvegicus*) model was approved by the Institutional Animal Ethics Committee for Research on Experimental Animals (Ref. No: IAEC No.01/01/10). Rats were maintained in an air-conditioned animal house (12 h dark: 12 h light and temperature 25±2°C), and provided with drinking water and pelleted diet (Lipton India Ltd., Mumbai, India) *ad libitum*. Female Wistar rats weighing 120 ± 10g with regular oestrous cyclicity, at proestrous phase were caged with male rats at a proportion of 2:1. Presence of sperm in vaginal plug/vaginal lavage on the next day morning confirmed successful mating, and the day was considered as Embryonic Day 1 (ED1). Each pregnant rat was placed in an individual cage, and received oral gavage of DEHP at three different doses 1, 10, 100 mg/kg b.wt./day in olive oil; the control animals received only vehicle (olive oil). Dose ranges used in this study corresponded with normal to occupational human exposure as reported previously[10] based on the average daily human exposure to phthalate, which was estimated to be between 1.7 and 52 μg/kg b.wt./day[22,23]. Each group consisted of six pregnant dams and these rats were treated from ED9 to 21 or until parturition. The day of parturition was designated as postnatal day 1 (PND1). The litter size was culled to four male offspring to avoid suckling and crowding effects. At PND 60 rats were anaesthetized with sodium thiopental

(40 mg/kg b.wt, i.p.), blood was collected, sera separated and used for the assay of T_3 and T_4. The thyroid tissue was cleaned of adhering tissues and blood, and used for testing the expression pattern of thyroid functional genes and proteins (six animals per treatment group belonging to different litters were used for assays).

2.2 Histology of Thyroid Glands

Immediately after anesthetization, the thyroid glands were removed and fixed in 4% paraformaldehyde (PFA) for 48 h and processed for paraffin embedding. Sections (5 µm thickness) obtained in a Leica microtome (Richmond Hill, Canada) were stained with hematoxylin and eosin and observed in a Nikon (Japan) microscope 80i. Network Internet System-imaging and capturing software (NIS) (Japan) was used for acquisition of images.

2.3 Hormone Assays

T_3 and T_4 levels in the serum were measured using a commercially available EIA Kit (Syntron Bioresearch, Inc, Carlsbad, CA). The sensitivity of the assay was 20ng/dl and 0.5 µg/dl for T_3 and T_4, respectively. The inter- and intra-assay variations of $T_3$and T_4 were 4.9-6.0% and 9.3-7.3%, and 10-8.5% and 11.9-6.1%, respectively.

2.4 Real-Time RT- PCR

Total RNA was extracted from thyroid tissue using TRIR reagent (ABgene, Surrey, UK) and quantified by measuring the absorbance at 260/ 280 nm. RNA with 1.8-1.9 ratio was pure. The integrity of the RNA was validated by running samples on 1% formaldehyde agarose gel. cDNA was synthesised from 2 µg of total RNA and oligo (dT) by using Omniscript® RT kit from Qiagen Inc (Valenica, USA) according to the manufacturer's protocol. The lists of primer sequences used are given in the table 1. Real-time RT-PCR was carried out in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, California). The reaction was performed using the MESA Green PCR Master Mix from Eurogentec (Seraing, Belgium) (which contains all the PCR components along with SYBR Green dye). The specificity of the amplification product was determined by melting curve analysis for each primer pair. The relative amount of each mRNA was normalised to β-actin. Data were analyzed by the comparative CT method and the fold change was calculated by the $2^{-\Delta\Delta CT}$ method (Schmittgen & Livak 2008) using CFX Manager Version 2.1 (Bio-Rad, Hercules, California).

2.5 Western Blot Analysis

The expression levels of TSHR, TRα and β, NIS, TPO, PDS, TTF-1, FOXE1, PAX8 and HHEX proteins were detected by western blotting as described earlier[24]. Tissue protein was extracted using Radio-Immune Precipitation Assay (RIPA) buffer (150 mM NaCl, 50M Tris, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS in 500 ml water) containing protease inhibitor cocktail; the homogenized samples were centrifuged at 12,000 x g for 10 min at 4ºC and the supernatant was collected and quantified using Folin-Ciacalteu phenol reagent, with crystalline Bovine Serum Albumin (BSA) as the standard. Total protein (75µg) was separated on 10% SDS-PAGE gel and electro-transferred onto a PVDF membrane (Millipore Biosciences Ltd., Billerica, MA, USA) at 100 V for 50 min. After the complete transfer of proteins, membranes were blocked overnight in blocking buffer containing 10% non-fat dry milk and incubated with specific primary antibodies for thyroidal proteins and transcription factors [TSHR (H-155), rabbit sc-13936; NIS (D-16), goat: sc-48052; TPO (MoAb47), mouse: sc-58432; PDS(E-20), goat: sc-16894; TTF-1 (8G7G3/1), mouse: sc-53136; FOXE1 (F-17), goat sc-16391); TRα (FL-408), rabbit: sc-772; TRβ (J52), mouse: sc-738; PAX8 (A-15), goat: sc-16279; HHEX (H-180), rabbit: sc-366054] were from Santa Cruz Biotechnology (California, USA) and β-actin (Sigma-Aldrich, St. Louis, USA) (H300-

Table 1. List of primers used in this study

Gene names	5'-Oligonucleotide	3'-Oligonucleotide	GeneBank accession numbers
Tshr	TCTCATTGCCTCCGTAGACC	AACTCGCTGGCAAAAACAGT	NM_012888.1
Tpo	GCATGCCTACCTTTCTACCG	GGAAGGAGGTCAAGCCATTC	NM_019353.2
Nis	GAGCAGACCATGGGGGTGCTC	AAGGAAGACACTGCCACCCTGA	U60282.1
Trα	AAGACGAGCAGTGTGTCGTG	GGTGGGATGGAGGTTCTTCTG	M18028
Trβ	GCTAGCCAAGAGGAAGCTGAT	TCTTGATGAGCTCCCATTCC	J03933.1
Pds	AAGTGAATGTCCCGAAGGTG	CATCGTCTTGAAGCAAGCA	NM_019214
18S	CGCTTCCTTACCTGGTTGAT	GAGCGACCAAAGGAACCATA	NM_001013237.2

aminoacids 76-375 of actin, humans; sc-10731) for 4 hr in 0.25% non-fat dry milk, at room temperature. The membranes were then washed twice with Tris-Buffer Saline (TBS) for 10 min each, incubated with HRP-conjugated goat-anti-rabbit/mouse secondary antibody (Santa Cruz Biotechnology, CA) at 1:15,000 dilution in 0.25% non-fat dry milk, incubated for 1 hr and washed twice with Tween-20 TBS and TBS for 10 min each. The membrane was exposed to Enhanced Chemi-Luminescence substrate (ECL) for 1 min (Chemi-luminescence kit; Pierce Biotechnology, Inc., Rockford, IL). The protein bands were quantified in a Bio-Rad Chemi Doc™ XRS Imaging system, using" Quantity One" software (Bio-Rad, Hercules, CA). The band intensity for protein was normalized with that of the internal control, β-actin, and expressed as optical density units relative to β-actin.

2.6 Statistical Analysis

Statistical analyses of data were performed using the Prism 6.00 Software (GraphPad Software for Windows, La Jolla, CA, USA) and values expressed as mean±S.E.M. Differences between groups were analysed by one-way Analysis Of Variance (ANOVA) followed by the Student Newman Keul's test for multiple post-hoc comparison tests. In all cases, $P<0.05$ was considered statistically significant.

3. Results

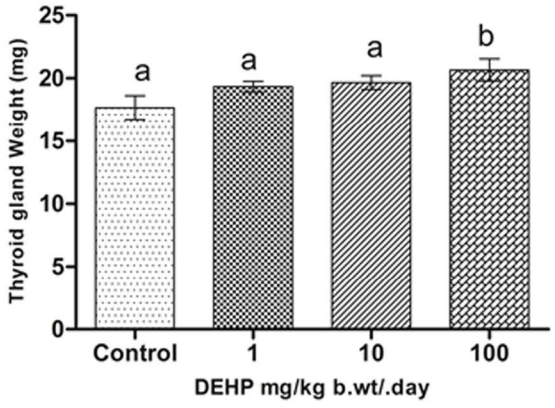

Figure 1. Effects of gestational DEHP exposure on absolute weight of thyroid gland in male offspring at PND 60. Each bar represents mean ± S.E.M. of six animals. Bars with same alphabet denote statistically insignificant difference between the respective means, while those with different alphabets denote statistically significant difference between such means at $p < 0.05$ level.

3.1 Thyroid Gland Weight

Gestational exposure to different dose of DEHP (1, 10 and 100 mg/kg b.wt./day), resulted in a significant increase in absolute thyroid gland weight in 100 mg DEHP-treated group, whereas no change was evident in 1mg and 10 mg DEHP-treated groups (Figure 1).

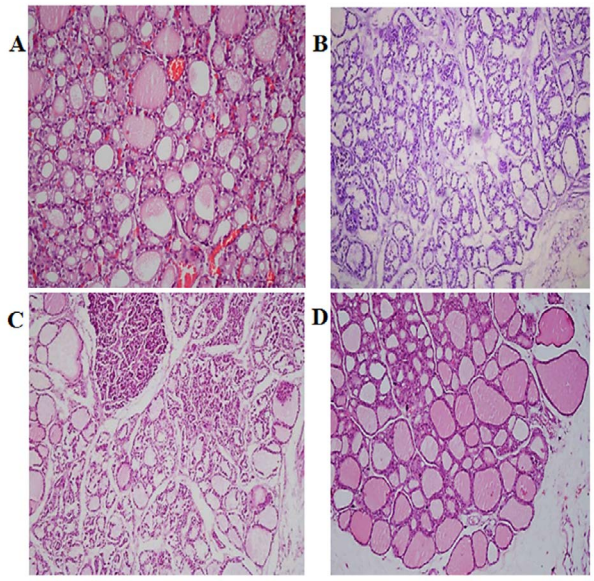

Figure 2. Histological representation of thyroid gland of rat exposed to gestational (GD 1 - GD 21) DEHP treatment (60 days) (20x) (20X). Microscopic representaton of thyroid gland - Control rat represents follicles lined by a sigle layer of cuboidal epithelium and lumen containing colloid. Disruption of follicular epithelium is evident in 1 and 10 mg DEHP treated rates. Rats exposed to 100 mg DEHP show large number of thyroid follicles filled with colloid. (A. Control, B- 1 mg DEHP, C- 10 mg DEHP, D- 100 mg DEHP/kg b.wt./day).

3.2 Histology of Thyroid Gland

Thyroid glands of control rats showed follicles lined by a cuboidal epithelium and filled with homogenous colloid, whereas rats belong to all experimental groups' revealed disruption of follicular epithelium. Accumulation of colloid was evident in follicles of rats exposed to100 mg DEHP (Figure 2).

3.3 Serum Hormone Titres

Serum T_3 (Figure 3(a)) and T_4 (Figure 3(b)) levels decreased significantly in puberal rats with gestational exposure to DEHP, when compared to coeval control rats, in a dose-dependent manner.

3.4 Expression Pattern of Tshr and Trα

Real-time RT- PCR and western blot data revealed increased expression of *Tshr* (Figure 4(a)) and *Trβ* (Figure 4(c)) in puberal rats with gestational exposure to DEHP, when compared to coeval control rats, whereas the expression of *Trα* (Figure 4(b)) decreased in these rats.

3.5 Expression Pattern of Genes Controlling Thyroid Hormone Synthesis

Gestational exposure to DEHP increased *Tpo* mRNA, whereas its protein decreased (Figure 5(a)). However, the levels of both mRNA and protein of *Nis* (Figure 5(b)) decreased indicating subnormal expression of *Nis* in puberal rats with gestational exposure to DEHP, when compared to coeval control rats. On the other hand, DEHP exposure increased the expression of *Pds* as indi-

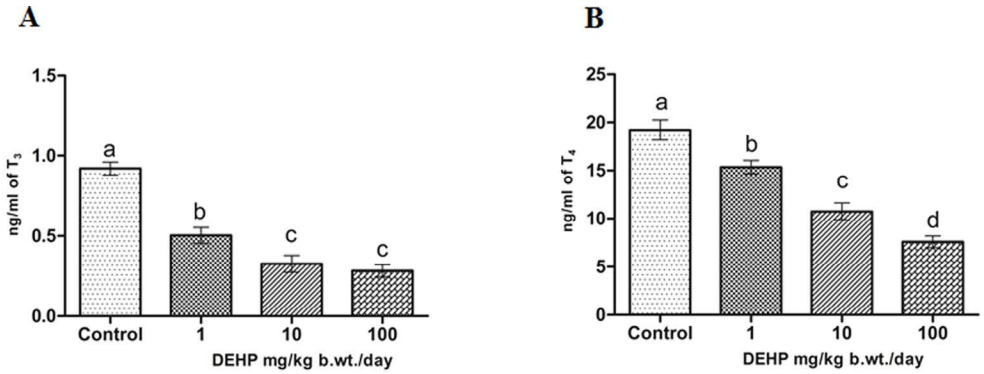

Figure 3. Effects of gestational DEHP exposure on serum T3 (A) and T4 (B) levels in male offspring at PND 60.. Each bar represents mean ± S.E.M. of six animals. Bars with same alphabet denote statistically insignificant difference between the respective means, while those with different alphabets denote statistically significant difference between such means at $p < 0.05$ level.

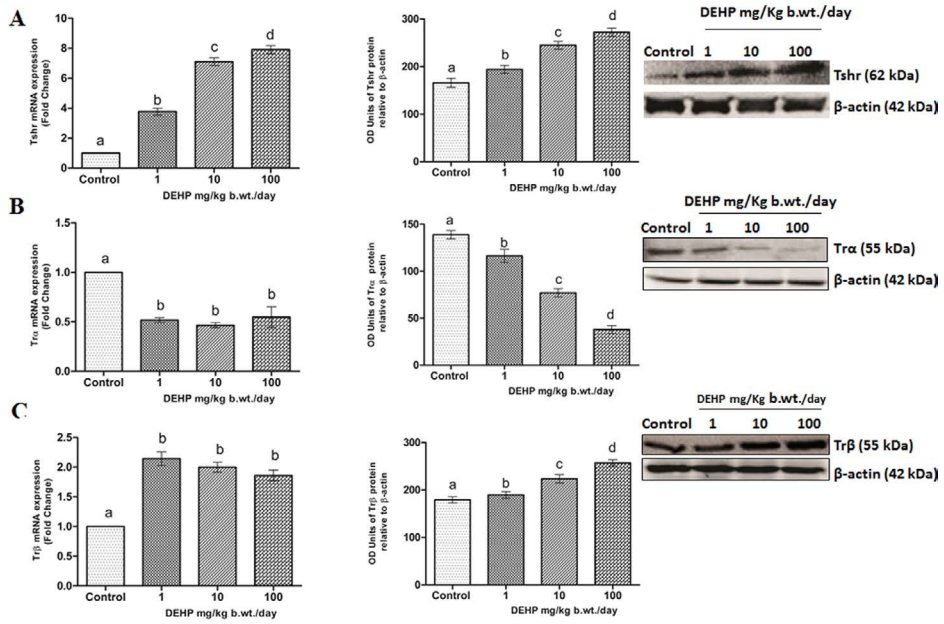

Figure 4. Effects of gestational DEHP exposure on expression of *Tshr* (A), *TrA* (B) and *TrB* (C) in the thyroid tissue of male offspring at PND 60. Each bar represents mean ± S.E.M. of three observations from pooled samples of six animals. Bars with same alphabet denote statistically insignificant difference between the respective means, while those with different alphabets denote statistically significant difference between such means at $p < 0.05$ level.

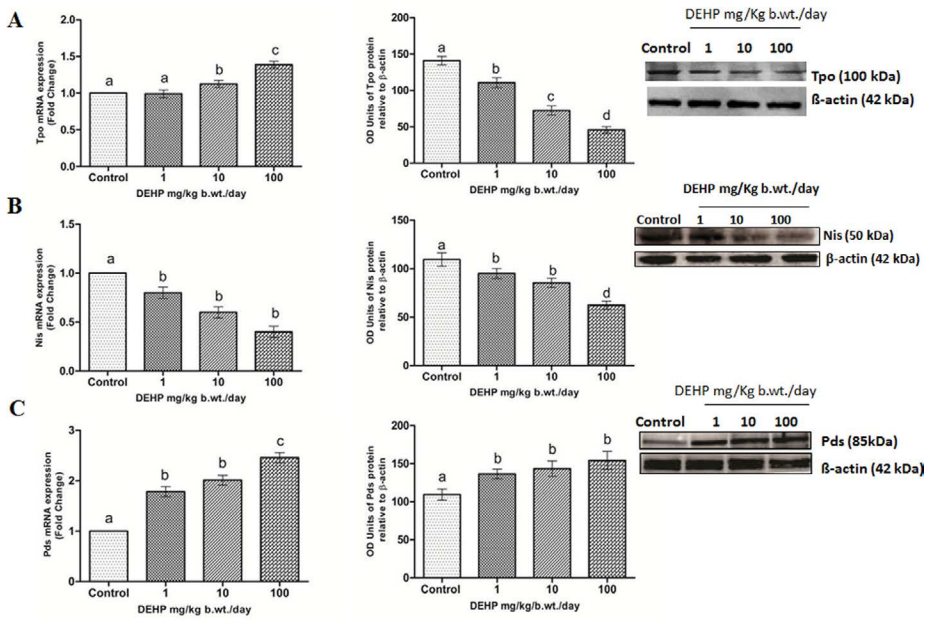

Figure 5. Effects of gestational DEHP exposure on gene expressions of Tpo (A), Nis (B), and Pds (C) in the thyroid tissue of male offspring at PND 60. Each bar represents mean ± S.E.M. of three observations from pooled samples of six animals. Bars with same alphabet denote statistically insignificant difference between the respective means, while those with different alphabets denote statistically significant difference between such means at $p < 0.05$ level.

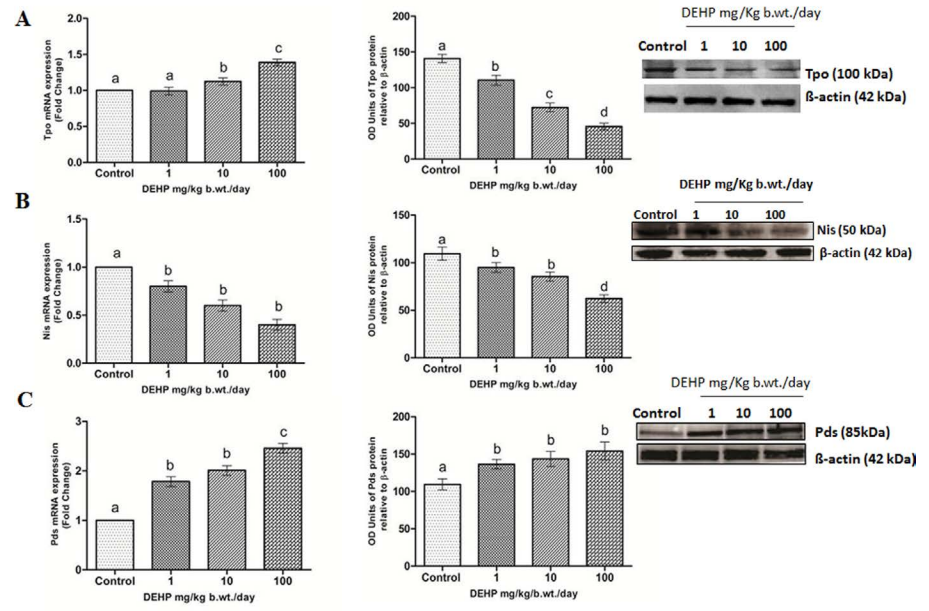

Figure 6. Effects of gestational DEHP exposure on the protein expressions of Ttf-1 (A), Foxe1 (B), Pax8 (©), and Hhex (D) in the thyroid tissue of male offspring at PND 60. Each bar represents mean ± S.E.M. of three observations from pooled samples of six animals. Bars with same alphabet denote statistically insignificant difference between the respective means, while those with different alphabets denote statistically significant difference between such means at $p < 0.05$ level.

cated by the consistent increase in the level of RT-PCR and western blot detection. (Figure 5(c)). Thus, there was a varied response of genes controlling iodothyronine synthesis, resulting in increased levels of Pds protein and decreased level of Nis and Tpo proteins.

3.6 Expression Pattern of Thyroid Specific Transcription Factors

Western blot detection revealed significantly increased expression level of Ttf-1 and Pax8 proteins in the DEHP-treated rats when compared to coeval control rats (Figure 6(a), (b)). On the other hand, Foxe1 and Hhex protein levels showed a dose-dependent decrease in all the DEHP-treated rats (Figure 6(c), (d)).

4. Discussion

Findings from the present study point out that gestational exposure to DEHP could lead to hypothyroidism in the progeny at puberal age. Our results also reveal a dose-dependent effect of DEHP, as the intensity of hypothyroidism was augmented with the increase in the dose of the chemical. Existing reports show that DEHP can disrupt the thyroid gland functions in men and experimental animals exposed to it postnatally[11-15]. To the best of our knowledge, this is the first report on the response of thyroid hormones to DEHP exposure during gestational period. An early study[18] reported an inverse association between MEHP and serum FT_4 levels in men, but not with TSH and T_3, after adjusting with other covariates. In contrast, MEHHP was significantly and positively associated with free T_4, but not with TSH and T_3, in a subgroup of 208/408 men. Subsequently, Meeker and Ferguson[19] studied 1,346 adults aged ≥20 years and 329 adolescents aged 12–19 years during 2007–2008 in a National Health and Nutrition Examination Survey (NHANES) and found a monotonic dose-dependent decrease in T_4 alone in relation to the urinary level of MEHHP. The above authors also observed a positive association between DEHP secondary metabolites (MEHP, MEHHP, Mono (2-Ethyl-5-Oxohexyl) Phthalate (MEOHP), Mono (2-Ethyl-5-Carboxypentyl) Phthalate (MECPP)) and total T_3 in adolescents. Sprague-Dawley (SD) rats (18 days old) given DEHP (0, 250, 500, 750 mg/Kg/day) for 30 days by oral gavage showed significant decrease in serum T_3, T_4 and TRH titres along with a decreasing trend in *Nis* and *Tpo* expression levels[25]. All the above reports attested the relation between DEHP/its metabolites and thyroid hormone status. Our findings point out that the progeny of mothers exposed to DEHP during pregnancy may be susceptible to hypothyroidism. To understand the molecular mechanism underlying DEHP-induced hypothyroid state, we investigated the response of genes that control iodothyronine synthesis.

Western blot detection of Tshr, the cell surface receptor for TSH, the primary trophic hormone that stimulates thyroid epithelial cell proliferation, and control the expression of differentiation markers such as *Tg, Tpo, Nis* necessary for the iodothyronine synthesis[26], point out that gestational exposure to DEHP might potentiate the action of TSH in the progeny. The synthesis and secretion of TSH, and expression of *Tshr* are repressed by T_3 through specific Trα, which has response element on *TSH* gene, whereas the parameters are up-regulated when there is a decrease or absence of T_3-mediated inhibition[27,28]. Therefore, augmented *Tshr* expression level observed in the present study, in response to all doses of DEHP tested, might be due to subnormal levels of circulating T_3 and T_4 along with decreased expression of *Trα,* a known negative regulator of Tshr[27,28].

Thus, it is clear that gestational exposure to DEHP interferes with the feedback control of iodothyronine synthesis. The varied responses of *Trα* and *Trβ* to gestational exposure to DEHP are on the expected lines as both these subtypes of TR are known to act in an opposite manner in different target organs/cells[29,30].

To gain further insight into the signalling of Tshr in DEHP-exposed rats, the responses of thyroid-specific target genes of TSH such as *Nis, Tpo* and *Pds* were tested. Thyroid hormone biosynthesis takes place in the follicular lumen, filled with colloid that is predominantly composed of Tg, which serves as the scaffold for hormone synthesis, apart from providing tyrosine molecules; uptake of iodide into the thyrocytes is facilitated by Nis, a glycoprotein anchored on the basal membrane, which mediates the active transport of I^- into the thyroid follicular cells[31,32]. The accumulated I^- is organified to molecular iodine (I^2) through the action of TPO, which catalyzes the oxidation of iodine, iodination of Tyr residues on Tg, and coupling of these iodo-tyrosine residues to form T_4 and T_3.[32] Thus, Nis and Tpo together control most of the key events related to iodothyronine synthesis. Therefore, puberal rats with gestational exposure to DEHP, which had decreased expression of *Tpo* and *Nis*, might have suffered from subnormal iodine transport, oxidation, iodination and coupling, leading to a hypothyroid state,

Wenzel et al.,[16] demonstrated increased uptake of iodide in a rat thyroid FRTL-5 cell line when challenged with non-cytotoxic doses of DEHP (100 μM to 1 mM), which was specifically due to augmentation of the *Nis*. Breous et al.,[17] did not find any increase in the expression of rat *Nis* mRNA or increase the activity of human *NIS* promoter constructs in response to DEHP. The inconsistency seen between these studies including the present *in vivo* study on puberal rats with gestational exposure to DEHP may be due to difference in the experimental protocol, models and doses of DEHP used. However, the present *in vivo* study clearly points out that there is under-expression of *Nis* gene in puberal rats with gestational exposure to DEHP. An interesting finding of the present study is the western blot detection of decreased Tpo protein level; despite an increase in its mRNA expression level in the progeny of mothers with gestational exposure to DEHP, suggesting a disposition between the rate of transcription and translation of *Tpo* or alteration in the stability of its mRNA and protein.

Increased expression of *Pds* in animals exposed to DEHP may be a compensatory mechanism to overcome repressed expression of *Nis*, and to maximally transport the available iodine from the intracellular compartment to the luminal compartment. Further studies on the uptake of labelled iodine at the basal plasma membrane and the transport of the same across the apical plasma membrane of thyrocytes may throw more light on this issue. We have also observed increased *Tg* mRNA expression in DEHP-treated rats of the present study (Data not shown). Probably, it is the outcome of augmented expression of *Tshr* and Ttf1. Iodinated *Tg* is stored in the follicular lumen of the thyroid and is released in response to specific stimulation by TSH[33].

To understand the response of specific transcription factors that control the expression of *Tshr, Tpo, Nis* and *Tg*, the expression levels of Ttf-1, Foxe1, Pax8 and Hhexin the thyroid were detected by western blot. Interestingly, our results on these thyroid-specific transcription factors point out a specific stimulatory effect of DEHP on Ttf-1 and Pax8 proteins while an inhibitory effect was evident in the expression levels of Foxe1 and Hhex proteins in puberal rat progeny with gestational exposure to DEHP. Ttf-1 is implicated as a critical regulatory component of *Tshr* gene and appears to be the link among TSH, Tshr and its downstream signalling molecules as well as transcription of *Tg* and *Tpo,* and, thus, biosynthesis of iodothyronines[34]. The consistent trend of increased expression of TTF-1protein along with the augmented levels of *Tshr, Tpo* and *Tg* mRNA, may point out the stimulatory effect of DEHP on the transcription of these thyroid specific genes because of its stimulatory effect on the expression of Ttf-1. However, data on T_4 and T_3 are not consistent with the expression level of above genes, suggesting that DEHP may affect synthesis of iodothyronines by interfering with some other key factor, masking the enhanced response of *Tshr, Tpo* and*Tg*. One of the interesting aspects of the present study is the decreased level of Tpo protein, despite an augmented response of its mRNA in DEHP-treated rats; probably, this has adversely affected iodothyronine synthesis, as discussed above. Ttf-1 can bind to *Nis* promoter region and regulate its expression too[35,36]. Studies have shown that Pax8 binding site is found in the far-upstream enhancer region of human *NIS* gene and required for the activation of its expression in the thyroid[37]. This suggests that gestational exposure to DEHP represses *Nis* expression through other factors like Foxe1 and Hhex, which recorded decreased expression level. Taken together, these results suggest that gestational exposure to DEHP brings about specific changes in different transcription factors controlling key genes controlling iodothyronine synthesis. Nevertheless, its adverse effect on Tpo and Nis proteins appears to be the key factor underlying the hypothyroid state of F1 progeny of mothers with gestational exposure to this endocrine disruptor. The response of *Tshr* gene may reflect negative feedback mechanism at the level of thyroid gland.

5. Conclusions

Findings from the present study support the proposed hypothesis and show for the first time that gestational exposure to the plasticizer DEHP may disrupt thyroid function in the progeny by modifying the expression of thyroid-specific genes that control iodothyronine synthesis and their transcription factors leading to hypothyroidism. It needs further studies to explain the inconsistency between mRNA and proteins of some genes.

6. Acknowledgment

The financial assistance from University Grants Commission (UGC) SAP-DRS III to the Department of Endocrinology, Dr. ALM PG Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai - 600113, is gratefully acknowledged. Critical

inputs from Prof. K. Balasubramanian and Dr. J. Arunakaran, Department of Endocrinology, University of Madras, are also gratefully acknowledged.

7. References

1. Chou K, Wright RO. Phthalates in food and medical devices. J Med Toxicol. 2006; 2:126–35. https://doi.org/10.1007/BF03161027 PMCid:PMC3550149
2. Cho SC, Bhang SY, Hong YC, Shin MS, Kim BN, Kim JW. Relationship between environmental phthalate exposure and the intelligence of school-age children. Environ Health Perspect. 2010; 118:1027–32. https://doi.org/10.1289/ehp.0901376 PMid:20194078 PMCid:PMC2920903
3. Shea KM. Pediatric exposure and potential toxicity of phthalate plasticizers. Pediatrics. 2003; 111(6 Pt 1):1467–74. https://doi.org/10.1542/peds.111.6.1467 PMid:12777573
4. Latini G. Monitoring phthalate exposure in humans. Clin Chim Acta. 2005; 361:20–9. https://doi.org/10.1016/j.cccn.2005.05.003 PMid:16004980
5. Silva MJ, Reidy JA, Herbert AR, Preau Jr JL, Needham LL, Calafat AM. Detection of phthalate metabolites in human amniotic fluid. Bull Environ Contam Toxicol. 2004; 72:1226–31. https://doi.org/10.1007/s00128-004-0374-4 PMid:15362453
6. Silva MJ, Samandar E, Preau Jr JL, Reidy JA, Needham LL, Calafat A. Automated solid-phase extraction and quantitative analysis of 14 phthalate metabolites in human serum using isotope dilution-high-performance liquid chromatography-tandem mass spectrometry. J Anal Toxicol. 2005; 29:819–24. https://doi.org/10.1093/jat/29.8.819 PMid:16374941
7. Zoeller RT. Environmental chemicals as thyroid hormone analogues: new studies indicate that thyroid hormone receptors are targets of industrial chemicals? Mol Cell Endocrinol. 2005; 242:10–5. https://doi.org/10.1016/j.mce.2005.07.006 PMid:16150534
8. Boas M, Feldt-Rasmussen U, Main KM. Thyroid effects of endocrine disrupting chemicals. Mol Cell Endocrinol. 2012; 355:240–8. https://doi.org/10.1016/j.mce.2011.09.005 PMid:21939731
9. Sekaran S, Jagadeesan A. In utero exposure to phthalate down regulates critical genes in Leydig cells of F1 male progeny. J Cell Biochem. 2015; 116:1466–77. https://doi.org/10.1002/jcb.25108 PMid:25649163
10. Rajesh P, Balasubramanian K. Gestational exposure to di(2-ethylhexyl) phthalate (DEHP) impairs pancreatic beta-cell function in F1 rat offspring. Toxicol Lett. 2015; 232:46–57. https://doi.org/10.1016/j.toxlet.2014.09.025 PMid:25280772
11. Poon R, Lecavalier P, Mueller R, Valli VE, Procter BG, Chu I. Subchronicoral toxicity of di-n-octyl phthalate and di(2-ethylhexyl) phthalate in the rat. Food Chem Toxicol. 1997; 35:225–39. https://doi.org/10.1016/S0278-6915(96)00064-6
12. Hinton RH, Mitchell FE, Mann A. Effects of phthalic acid esters on the liver and thyroid. Environ Health Perspect. 1986; 70:195–210. https://doi.org/10.1289/ehp.8670195 PMid:3830106 PMCid:PMC1474287
13. Price SC, Chescoe D, Grasso P, Wright M, Hinton RH. Alterations in the thyroids of rats treated for long periods with di-(2-ethylhexyl) phthalate or with hypolipidaemic agents. Toxicol Lett. 1988; 40:37–46. https://doi.org/10.1016/0378-4274(88)90181-6
14. ATSDR. Toxicological profile for di(2-Ethylhexyl) Phthalate. Agency for Toxic Substances and Disease; 2002.
15. Howarth JA, Price SC, Dobrota M, Kentish PA, Hinton RH. Effects on male rats of di-(2-ethylhexyl) phthalate and di-n-hexylphthalate administered alone or in combination. Toxicol Lett. 2001; 121:35–43. https://doi.org/10.1016/S0378-4274(01)00313-7
16. Wenzel A, Franz C, BreousE, Loos U. Modulation of iodide uptake by dialkylphthalate plasticizers in FRTL-5 rat thyroid follicular cells. Mol Cell Endocrinol. 2005; 244: 63–71. https://doi.org/10.1016/j.mce.2005.02.008 PMid:16289305
17. Breous E, Wenzel A, Loos U. The promoter of the human sodium/iodide symporter responds to certain phthalate plasticisers. Mol Cell Endocrinol. 2005; 244:75–8. https://doi.org/10.1016/j.mce.2005.06.009 PMid:16257484
18. Meeker JD, Calafat AM, Hauser R. Di-(2-ethylhexyl) phthalate metabolites may alter thyroid hormone levels in men. Environ Health Perspect. 2007; 115:1029–34. https://doi.org/10.1289/ehp.9852 PMid:17637918 PMCid:PMC1913587
19. Meeker JD, Ferguson KK. Relationship between urinary phthalate and bisphenol A concentrations and serum thyroid measures in US adults and adolescents from the National Health and Nutrition Examination Survey (NHANES) 2007–2008. Environ Health Perspect. 2011; 119:1396–402. https://doi.org/10.1289/ehp.1103582 PMid:21749963 PMCid:PMC3230451
20. Van dartel DA, Pennings JL, Hendriksen PJ, Van schooten FJ, Piersma AH. Early gene expression changes during embryonic stem cell differentiation into cardiomyocytes and their modulation by monobutyl phthalate. Reprod Toxicol. 2009; 27:93–102. https://doi.org/10.1016/j.reprotox.2008.12.009 PMid:19162170
21. Damante G, Tell G, DiLauro R. A unique combination of transcription factors controls differentiation of thyroid cells. Prog Nucleic Acid Res Mol Biol. 2001; 66:307–56. https://doi.org/10.1016/S0079-6603(00)66033-6

22. Francis-Lang H, Zannini M, De Felice M, Berlingieri MT, Fusco A, DiLauro R. Multiple mechanisms of interference between transformation and differentiation in thyroid cells. Mol Cell Biol. 1992; 12:5793–800. https://doi.org/10.1128/MCB.12.12.5793 PMid:1448106 PMCid:PMC360519
23. Koch HM, Drexler H, Angerer J. An estimation of the daily intake of Di(2-Ethylhexyl)Phthalate (DEHP) and other phthalates in the general population. Int J Hyg Environ Health. 2003; 206:77–83. https://doi.org/10.1078/1438-4639-00205 PMid:12708228
24. Frederiksen H, Aksglaede L, Sorensen K, Skakkebaek NE, Juul A, Andersson AM. Urinary excretion of phthalate metabolites in 129 healthy Danish children and adolescents: Estimation of daily phthalate intake. Environ Res. 2011; 111:656–63. https://doi.org/10.1016/j.envres.2011.03.005 PMid:21429484
25. Aruldhas MM, Ramalingam N, Jaganathan A, Sashi AMJ, Stanley JA, Nagappan AS, Vasavan J, Kannan A, Seshadri VN. Gestational and neonatal-onset hypothyroidism alters androgen receptor status in rat prostate glands at adulthood. Prostate. 2010; 70:689–700. PMid:20033886
26. Liu C, Zhao L, Wei L, Li L. DEHP reduces thyroid hormones via interacting with hormone synthesis-related proteins, deiodinases, transthyretin, receptors, and hepatic enzymes in rats. Environ Sci Pollut Res Int. 2015; 22:12711–9. https://doi.org/10.1007/s11356-015-4567-7 PMid:25913319
27. Stuart A, Oates E, Hall C, Grumbles R, Fernandez L, Taylor N, Puett D, Jin S. Identification of a point mutation in the thyrotropin receptor of the hyt/hyt hypothyroid mouse. Mol Endocrinol. 1994; 8:129–38. https://doi.org/10.1210/mend.8.2.8170469 https://doi.org/10.1210/me.8.2.129
28. Shupnik MA, Ridgway EC, Chin WW. Molecular biology of thyrotropin. Endocr Rev. 1989; 10:459–75. https://doi.org/10.1210/edrv-10-4-459 PMid:2693083
29. Roelfsema F, Veldhuis JD. Thyrotropin secretion patterns in health and disease. Endocr Rev. 2013; 34:619–57. https://doi.org/10.1210/er.2012-1076 PMid:23575764
30. Ishihara A, Sawatsubashi S, Yamauchi K. Endocrine disrupting chemicals: Interference of thyroid hormone binding to transthyretins and to thyroid hormone receptors. Mol Cell Endocrinol. 2003; 199:105–17. https://doi.org/10.1016/S0303-7207(02)00302-7
31. Sugiyama S, Shimada N, Miyoshi H, Yamauchi K. Detection of thyroid system-disrupting chemicals using in vitro and in vivo screening assays in *Xenopus laevis*. Toxicol Sci. 2005; 88:367–74. https://doi.org/10.1093/toxsci/kfi330 PMid:16179385
32. Bizhanova A, Kopp P. Minireview: The sodium-iodide symporter NIS and pendrin in iodide homeostasis of the thyroid. Endocrinology. 2009; 150:1084-90. https://doi.org/10.1210/en.2008-1437 PMid:19196800 PMCid:PMC2654752
33. Kohn LD, Suzuki K, Nakazato M, Royaux I, Green ED. Effects of thyroglobulin and pendrin on iodide flux through the thyrocyte. Trends Endocrinol Metab. 2001; 12:10-16. https://doi.org/10.1016/S1043-2760(00)00337-4
34. Lin JD. Thyroglobulin and human thyroid cancer. Clin Chim Acta. 2008; 388:15–21. https://doi.org/10.1016/j.cca.2007.11.002 PMid:18060877
35. Kohn LD, Shimura H, Shimura Y, Hidaka A, Giuliani C, Napolitano G, Ohmori M, Laglia G, Saji M. The thyrotropin receptor. Vitamins and Hormones. 1995; 50:287–384. https://doi.org/10.1016/S0083-6729(08)60658-5
36. Kambe F and Seo H. Thyroid-specific transcription factors. J of Endocrinol. 1997; 44:775–84. https://doi.org/10.1507/endocrj.44.775
37. Dohan O, De la Vieja A and Paroder V. Molecular analysis of the sodium/iodide symporter: Characterization, regulation, and medical significance. Endocr Rev. 2003; 24:48–77. https://doi.org/10.1210/er.2001-0029 PMid:12588808
38. Taki K, Kogai T, Kanamoto Y, Hershman JM, Brent GA. A thyroid-specific far-upstream enhancer in the human sodium/ iodide symporter gene requires Pax-8 binding and cyclic adenosine 3',5'-monophosphate response element-like sequence binding proteins for full activity and is differentially regulated in normal and thyroid cancer cells. Mol Endocrinol. 2002; 16:2266–82. https://doi.org/10.1210/me.2002-0109 PMid:12351692