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Evaluation of Sex hormones in male and female Egyptian population naturally exposed to Dibutyl phthalate: a cross-sectional study

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ABSTRACT

Endocrine-disrupting chemicals (EDC) an exogenous chemical or mixture of chemicals, which can interfere with hormonal action. The phthalates and their metabolism like Dibutyl phthalate (DBP) are considered EDCs disturb the action of reproductive hormones. A cross-sectional study was conducted on 84 selected adult volunteers from both genders to assess the association between serum DBP concentration and sex hormone levels in adult Egyptians. Serum DBP was measured by Gas chromatography-mass spectrometer. Serum sex hormones, sex hormone-binding globulin (SHBG), and Thyroid-stimulating hormone (TSH) were measured by enzyme-linked immunosorbent assay. In males, there was a significant decrease in total testosterone (T), Free testosterone (FT), Bioavailable testosterone (BT), Free Androgen Index (FAI) between low and high exposure to DBP, while Estradiol (E2) was significantly increased. In the female, there were significant increases in total E2, Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH), and significant reduction in progesterone in high exposure group compared to low exposure to DBP. This study showed that there was a significant strong correlation between DBP with T, T/ E2 in males while female subjects showed a significant strong positive correlation with LH and FSH. There is increasing evidence that DBP has an impact on human sex hormones which can cause many physiological disturbances and many diseases in Egyptian adults. It is recommended that reduce human exposure to DBP in the food supply.

Introduction

The Endocrine Society states an endocrine-disrupting chemical (EDC) their biological effect may vary considerably. Many EDCs act as estrogenic / anti-androgenic compounds, mimicking the activities of either the estrogen receptor (ER) or the androgen receptor (AR), ultimately inducing cell apoptosis, proliferation, differentiation, carcinogenesis, and inflammation [1]. Xenoestrogens are manmade chemicals that resemble natural estrogens. These chemical compounds mimic, hormone-like properties, and impact the flesh by continuous interaction with natural estrogens, thus, when these chemicals stay within the body, they might cause serious human health concerns [2]. Phthalates are a very important class of EDCs with very high usage within the industry as plasticizers and have adverse health effects.

The widespread use of phthalate containing products inside or outside homes leads to continuous exposure through air, water, food, and absorption through the skin which has been reported in several bio- monitoring studies with detection in urine, serum, milk, and other body fluids [3-5].

Phthalate esters are classified into high molecular weight phthalates, like di-2-Ethylhexyl phthalate (DEHP), while the low mass phthalates, like diethyl phthalate (DEP), dibutyl phthalate (DBP), and butyl benzyl phthalate (BBP). Phthalates became global contaminants due to leaching from their widespread applications thus, phthalates can migrate from packing material to food. Human exposure to phthalates has been an increased worry due to the findings of many toxicology studies in animals [6]. Epidemiological and experimental studies have also revealed that exposure to many phthalates compounds can alter sex steroid levels in human subjects which causes adverse effects on the development and performance of the reproductive system [7 - 9].

DBP is one of every of the foremost widely studied phthalate esters that disturb the expansion of normal reproductive organs. DBP is an endocrine disruptor, because it prevents the assembly and action of the many hormones [10]. Dibutyl phthalate may be a substance that may disrupt hormonal balance, named as EDCs which might cause fertility disorders, genital development disorders, hormone dependent cancer (e.g., breast, prostate, ovary or testicle cancer), damage to the fetus nervous system, and metabolism disorders [11]. Because DBP is bound non-covalently to their products, it can easily leach from these plastics and, consequently, be ingested. Therefore, the overall population is exposed to phthalates through consumer products, diet, and medical devices. Oxidative stress is one of the mechanisms related to phthalate esters prompted tissue atrophy in several tissues. These involve causing alterations within the antioxidant defense balance of tissues yet as inducing per oxidative damages [12]. DBP is significantly related with reproductive health and disrupts gonadal steroidogenesis. As demonstrated by previous studies, the primary endocrine-disrupting mechanism of phthalates is to act as anti-androgens, but DBP also has an estrogenic property. So, it can alter the synthesis and releases of reproductive hormones [13]. Within the present century, continuous human exposure to materials like endocrine-disrupting chemicals has been considered as a growing global health risk.

The aim of this study is to evaluate sex hormones in male and female Egyptian population which naturally exposed to Dibutyl phthalate. Unfortunately, there were no studies published to assess the DBP in the Egyptian population and its effect on their health especially sex hormones.

Material and methods

The study was adapted to a cross-sectional population and was conducted on (84) selected adult volunteers from both genders (40 male and 44 female). Selection of volunteers was according to certain criteria as following: inclusion criteria was (Age females 20-40 and males 30-50 years old, generally good healthy) while exclusion criteria was pregnant and lactating women, debit patients, liver diseases patients, history of moderate to severe brain injury and past or present use of hormonal therapy and chemotherapy. Ethical consideration the current study was approved by the Research and Ethics Review Committee of the General Organization for Teaching Hospitals and Institutes (NO: IN000090) by date 27/3/2017. All the participants gave their informed consent.

Venous blood was drawn and collected into a plain tube. After clotting, the samples were centrifuged, and the serum was separated and collected at many vials. The serum was stored at -80°C until the analysis was performed. The studied samples were categorized into two groups according to the sex while two subgroups according to DBP exposure level to low exposure level which considered as Negative control group (not detect DBP in serum sample) and high exposure level (detected DBP in serum samples).

Serum total testosterone (T), Serum-free testosterone (FT), Luteinizing Hormone (LH), Follicle – Stimulating Hormone (FSH), Prolactin (PRL), Estradiol (E2), Progesterone, Sex hormone-binding globulin (SHBG) and Thyroid-stimulating hormone (TSH) were measured by enzyme-linked immunosorbent assay (ELISA) according to manual instructions of Chemux Bioscience, inc U.S.A. The Free Androgen Index (FAI) was calculated as a total T _ 100/SHBG according to Morris *et al.* [15]. Also, bioavailable testosterone (BT) was calculated as BT = [FT * (1+ ((K_A x Alb)/ molecular weight Alb)] (K_A is constant =3.6x10^4) according to Vermeulene *et al.* [16].

Serum DBP residue was measured gas chromatography–mass spectrometry detector according to Colón *et al.* [14] method. Serum proteins was predicted by adding 1 mL acetonitrile to 1.0 mL sample. Centrifuged the sample and transfer the supernatant into another clean stripped glass tube.

Five milliliters of the extraction mixture, which consisted of an 8:1 solution of hexane and dichloromethane, was added to each sample, then submitted to ultrasound extraction for 5 min in an Ultrasonic apparatus (Fisher Scientific Bransonic).

The phases were allowed to separate, and the extract was transferred to a centrifuge tube. The extraction procedure was performed twice and then cleaned up by using solid-phase extraction (SPE) technique all eluents were dried under nitrogen. The dried sample was re-dissolved in 1 mL of hexane for the (GC–MS Agilent 6890N Network GC System coupled with a model 5973Network quadruple mass spectrometer) by using GC Column(HP-5 30m, 0.32mm, 0.25µm).

The concentrated extract (1 µL) was injected into the GC/MS system. The samples were heated to an initial temperature of 70°C for 4 min and then raised to 130°C at 5°C/min and then to 250°C at a rate of 10°C/min.

Relative area of the peaks was compared for each standard to the area of standards in a calibration curve generated under the same conditions and calculated the concentration. All Reagents and chemicals were of pesticide residue grade and Standards of DBP (purity, 98.0%) were obtained from Sigma-Aldrich.

Statistical analysis

Data analysis was performed using SPSS software version 21.0 (SPSS, Inc.). Results are expressed as Mean ± SD or as median plus 25th, 75th percentile according to variable distributions according to normality test (Shapiro-Wilk test). Differences between groups were analyzed by independent

Student t-test or Mann–Whitney U-test when appropriate. Correlations between variables were performed using Spearman's rho correlation coefficient.

Results

The studied population includes 84 adults (40 male + 44 female), whose male's ages ranged from 30 to 50 years while female's ages ranged from 20 to 40 according to the birding period for each gender. According to the normality test, the normally distributed parameters were represented by mean ± SD while not normally distributed were represented by median and 25th, 75th percentile.

Table 1 showed all biochemical parameters to investigate that all subjects meet study Inclusion Criteria. Serum DBP median concentrations among the studied males' subjects was 0.325 µg/L and the interquartile range was 0.30 – 0.34 µg/L while, in females the median DBP was 0.326 µg/L, and the interquartile range was 0.28 –0.38 µg/L. According to the serum DBP detection limits, low exposure subject whose DBP level does not detect is considered as Negative control.

Table 1 Biochemical characteristics of the studied subjects

SEX	Male (N = 40)	Female (N = 44)
Parameter *	Mean ± SD median (25th,75th)	Mean ± SD median (25th,75th)
TSH (µIU/ml)	0.91 (0.63-1.9)	1.2 (0.79-2.2)
Testosterone (ng/ml) #	12.1 ± 3.6	2.8 ± 1.58
Estradiol (pg/ml) #	61.8 ± 8.3	69.4 ± 9.5
Progesterone (ng/ml)	1.41 (0.54-2.7)	1.37 (0.51-3.6)
LH (µIU/ml)	7.3 (5.8-9.5)	9.5 (6.3-14.1)
FSH (µIU/ml)	2.4 (1.5-4.0)	3.9 (2.8-6.6)
Prolactin (ng/ml)	8.4 (6.7-10.6)	12.5 (8.5-18.6)
sBG (nmol/L)	46.5 (39.2-57.4)	40.4 (29.2-59.7)
free testosterone (ng/dL) #	0.20 ± 0.07	0.0383 ± 0.03
Bioactive Testosterone (ng/dL) #	4.7 ± 1.75	0.9 ± 0.65
Free androgen index (%) #	121 ± 52.1	21.83 ± 18.1
T/E2 ratio #	1.9 ± 0.49	0.41 ± 0.13
Dibutyl phthalate µg/L	0.325 (0.30 – 0.34)	0.326 (0.28 – 0.38)

Parametric variables

** DBP was detected in 21(47%) female subject and 17 (43%) male subjects

The subjects were divided to two sub-groups: low exposure of DBP (23 male & 23 female) and high exposure of DBP (17 male & 21 female). Data in Table 2 cleared that DBP metabolite was detected in seventeen male subjects, which causes a significant reduction in T, FT, BT, FAI, and T/E2 ratio levels, while FSH and E2 levels were significantly increased as compared to male subjects with low exposure level of DBP. From Table 3, it was observed that about 21 females were highly exposed to DBP, there was a significant elevation in E2, FSH and LH levels in those subject as compared to low expose subjects. From Table 4 and Figs. 1 & 2, it was observed that there was a significant negative strong correlation between total testosterone, T/E2 Ratio, and moderate correlation FT, BT with DBP metabolite in male subjects, while E2 showed moderate inverse correlation. In female subjects, there was a direct strong significant correlation between LH, FSH with DBP metabolite. DBP concentration was detect and calculate by using Gc-Ms chromatogram according to Fig. 3.

Table 2 Comparison between Biochemical characteristics and Sex hormone levels in High exposed and Low exposed to DBP Male subjects

Groups	low exposure of DBP	High exposure of DBP	*P-value*
Parameter	N= 23	N= 17	
TSH (µIU/ml)	0.844 (0.65 – 1.91)	1.00 (0.57 – 2.2)	0.830
Testosterone (ng/ml)	15.3 (14.1 – 16.2)	9.8 (8.13 – 11.2)	0.000**
Estradiol (pg/ml)	57.5 (54.3 – 62.0)	64.7 (60.3 – 70.6)	0.015*
Progesterone (ng/ml)	1.28 (0.55 – 3.6)	1.61 (0.46 – 5.21)	0.809
LH (µIU/ml)	7.17 (5.9 – 9.5)	7.4 (5.6 – 9.6)	0.694
FSH (µIU/ml)	1.76 (1.16 – 3.17)	2.78 (1.84 – 6.7)	0.046*
Prolactin (ng/ml)	7.74 (5.9 – 10.3)	8.5 (6.9 – 11.1)	0.401
SHBG (nmol/L)	49.0 (40.9 – 67.7)	45.9 (38.2 – 52.5)	0.524
FT (ng/dL)	0.237 (0.2 – 0.31)	0.161(0.15 – 0.18)	0.002**
BT (ng/dL)	6.0 (5.14 – 6.74)	3.71 (3.3 – 4.2)	0.000**
FAI (%)	147.8 (122.7 – 183.7)	91.03 (74.8 – 118.8)	0.001**
T/E2 ratio	2.3 (1.9 – 2.4)	1.53(1.3 – 1.8)	0.000**

**P ≤ 0.05* and ***P ≤ 0.001* was considered significant and highly significant, respectively

Table 3 Comparison between Biochemical characteristics and Sex hormone levels in High exposed and Low exposed to DBP Female subjects

Groups	low exposure of DBP	High exposure of DBP	*P-value*
Parameter	N= 23	N= 21	
TSH (µIU/ml)	1.13 (0.77 – 2.16)	1.5 (0.81 – 2.84)	0.613
Testosterone (ng/ml)	2.9 (1.1 – 3.99)	2.78 (1.63 – 4.24)	0.718
Estradiol (pg/ml) #	62.79 ± 17.7	79.88 ± 17.9	0.003##**
Progesterone (ng/ml)	1.66 (0.93 – 2.68)	0.87 (0.54 – 2.4)	0.027*
LH (µIU/ml)	9.1 (6.84 – 10.3)	13.4 (6.2 – 34.1)	0.038*
FSH (µIU/ml)	3.59 (2.78 – 5.24)	6.6 (4.1 – 12.1)	0.00**
Prolactin (ng/ml)	12.6 (9.47 – 17.1)	12.4 (6.82 – 21.1)	0.462
SHBG (nmol/L)	39.59 (26.6 – 51.5)	40.95 (29.5 – 62.5)	0.531
FT (ng/dL)	0.037 (0.018 – 0.053)	0.030 (0.02 – 0.056)	0.838
BT (ng/dL)	0.78 (0.38 – 1.2)	0.77 (0.54 – 1.3)	0.713
FAI (%)	16.1 (8.9 – 28.3)	19.1 (11.4 – 28.3)	0.673
T/E2 ratio	0.46 (0.27 – 0.60)	0.32 (0.2 0 – 0.46)	0.106

Parametric variables, ## Student T-test P-value

* $P \leq 0.05$ and ** $P \leq 0.001$ was considered significant and highly significant, respectively

Table 4 Correlation between sex hormones and DBP concentrations in both gender

SEX	Male		Female	
Parameters	*R-Value*	*P-value*	*R-Value*	*P-value*
TSH (µIU/ml)	0.229	0.319	- 0.127	0.628
Testosterone (ng/ml)	- 0.867**	0.000	- 0.185	0.477
Estradiol (pg/ml)	0.443*	0.045	0.189	0.467
Progesterone (ng/ml)	0.058	0.802	0.430	0.058
LH (µIU/ml)	- 0.020	0.931	0.724**	0.001
FSH (µIU/ml)	0.289	0.203	0.713**	0.001
Prolactin (ng/ml)	- 0.124	0.591	- 0.226	0.383
SHBG (nmol/L)	0.219	0.341	- 0.285	0.268
FT (ng/dL)	- 0.584*	0.045	0.102	0.697
BT (ng/dL)	- 0.496-*	0.022	- 0.127	0.628
FAI (%)	- 0.369	0.099	- 0.074	0.779
T/E2 ratio	- 0.713-**	0.000	- 0.259	0.315

** P ≤ 0.05* and *** P ≤ 0.001* was considered significant and highly significant, respectively

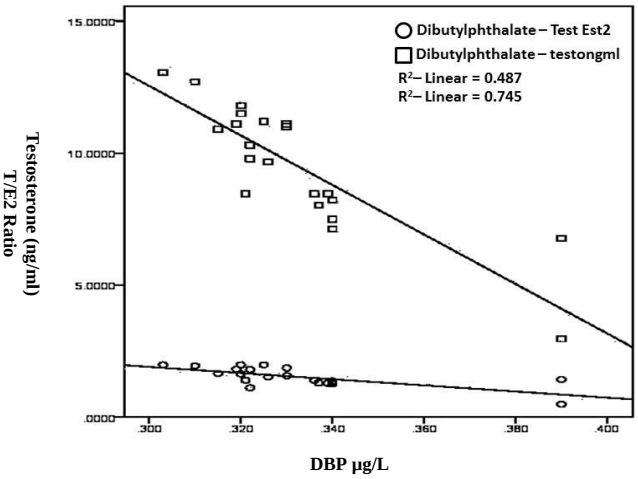


Fig. 1 Correlation between testosterone and T/E2 ratio levels and DBP level in male subjects

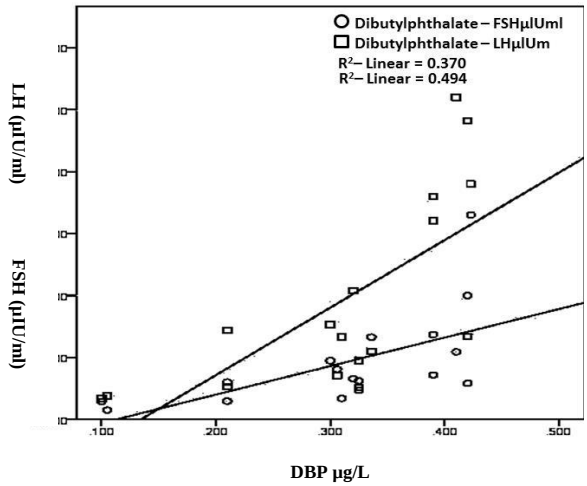


Fig. 2 Correlation between FSH, LH levels in females and DBP level in female subjects

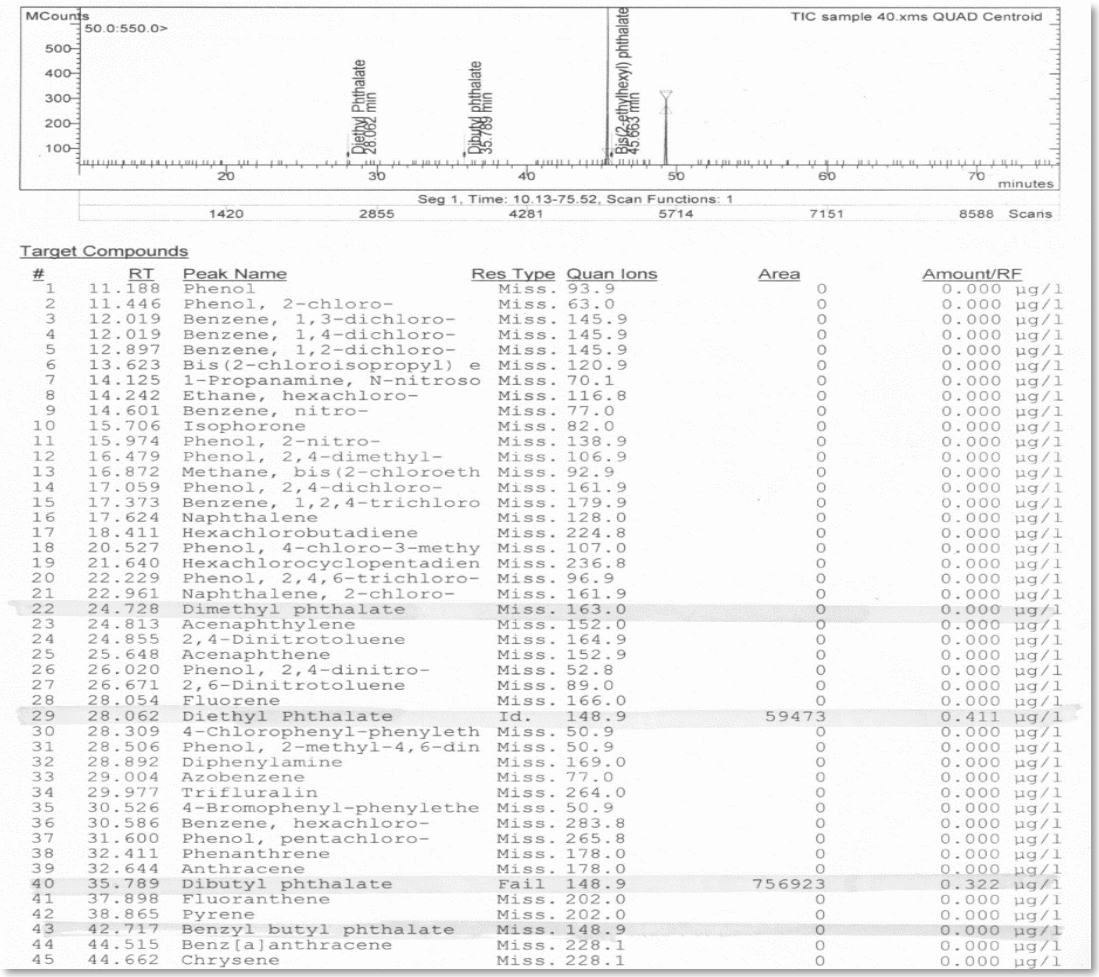
Discussion

Experimental and observational studies support a connection between exposure to phthalate esters and endocrine function, particularly steroid hormone dysregulation. Evidence suggests that phthalates may have anti-androgenic effects, such as reduced testosterone production and bioavailability, decreased sperm production and motility, shortened anogenital distance, and increased odds of genital anomalies [17,18].

Phthalates can interfere with the concentrations, signaling, and/or functions of sex hormones which altered reproductive hormone levels among adolescents and adults, pregnant women, and newborns. That was found to be negatively associated with concentrations of different hormones such as FSH, LH, TSH, T, T4, T3, DHEA-S, SHBG, AMH, and inhibin B in blood. Additionally, Epidemiologic studies also suggested that phthalates may adversely affect the thyroid hormone's blood level. However, the direction and the implicated phthalates were inconsistent across studies [4,19]. However, DBP effect on thyroid doesn't observed in this study in both gender and this result was in agreement with ***Donat-Vargas et al.*** as reported that there was no significant relationship was found between TSH and phthalate metabolites [20].

This study cleared that estradiol, FSH, and LH were significantly affected by DBP exposure. FSH and LH were positively associated with DBP level in female subjects that results agree with previous work showing disruptions in gonadotropin levels, and steroidogenesis that may be due to estrogenic and anti-androgenic effects of DBP [21 - 23].

DBP may affect the steroidogenic capacity in human ovaries and effects on the female reproductive system as it can alter the synthesis and bioactivity of aromatase which enzyme responsible for a key step in the biosynthesis of estrogens [13].



Target Compounds

#	RT	Peak Name	Res Type	Quan Ions	Area	Amount/RF
1	11.188	Phenol	Miss.	93.9	0	0.000 µg/l
2	11.446	Phenol, 2-chloro-	Miss.	63.0	0	0.000 µg/l
3	12.019	Benzene, 1,3-dichloro-	Miss.	145.9	0	0.000 µg/l
4	12.019	Benzene, 1,4-dichloro-	Miss.	145.9	0	0.000 µg/l
5	12.897	Benzene, 1,2-dichloro-	Miss.	145.9	0	0.000 µg/l
6	13.623	Bis(2-chloroisopropyl) e	Miss.	120.9	0	0.000 µg/l
7	14.125	1-Propanamine, N-nitroso	Miss.	70.1	0	0.000 µg/l
8	14.242	Ethane, hexachloro-	Miss.	116.8	0	0.000 µg/l
9	14.601	Benzene, nitro-	Miss.	77.0	0	0.000 µg/l
10	15.706	Isophorone	Miss.	82.0	0	0.000 µg/l
11	15.974	Phenol, 2-nitro-	Miss.	138.9	0	0.000 µg/l
12	16.479	Phenol, 2,4-dimethyl-	Miss.	106.9	0	0.000 µg/l
13	16.872	Methane, bis(2-chloroeth	Miss.	92.9	0	0.000 µg/l
14	17.059	Phenol, 2,4-dichloro-	Miss.	161.9	0	0.000 µg/l
15	17.373	Benzene, 1,2,4-trichloro	Miss.	179.9	0	0.000 µg/l
16	17.624	Naphthalene	Miss.	128.0	0	0.000 µg/l
17	18.411	Hexachlorobutadiene	Miss.	224.8	0	0.000 µg/l
18	20.527	Phenol, 4-chloro-3-methy	Miss.	107.0	0	0.000 µg/l
19	21.640	Hexachlorocyclopentadien	Miss.	236.8	0	0.000 µg/l
20	22.229	Phenol, 2,4,6-trichloro-	Miss.	96.9	0	0.000 µg/l
21	22.961	Naphthalene, 2-chloro-	Miss.	161.9	0	0.000 µg/l
22	24.728	Dimethyl phthalate	Miss.	163.0	0	0.000 µg/l
23	24.813	Acenaphthylene	Miss.	152.0	0	0.000 µg/l
24	24.855	2,4-Dinitrotoluene	Miss.	164.9	0	0.000 µg/l
25	25.648	Acenaphthene	Miss.	152.9	0	0.000 µg/l
26	26.020	Phenol, 2,4-dinitro-	Miss.	52.8	0	0.000 µg/l
27	26.671	2,6-Dinitrotoluene	Miss.	89.0	0	0.000 µg/l
28	28.054	Fluorene	Miss.	166.0	0	0.000 µg/l
29	28.062	Diethyl Phthalate	Id.	148.9	59473	0.411 µg/l
30	28.309	4-Chlorophenyl-phenyleth	Miss.	50.9	0	0.000 µg/l
31	28.506	Phenol, 2-methyl-4,6-din	Miss.	50.9	0	0.000 µg/l
32	28.892	Diphenylamine	Miss.	169.0	0	0.000 µg/l
33	29.004	Azobenzene	Miss.	77.0	0	0.000 µg/l
34	29.977	Trifluralin	Miss.	264.0	0	0.000 µg/l
35	30.526	4-Bromophenyl-phenylethe	Miss.	50.9	0	0.000 µg/l
36	30.586	Benzene, hexachloro-	Miss.	283.8	0	0.000 µg/l
37	31.600	Phenol, pentachloro-	Miss.	265.8	0	0.000 µg/l
38	32.411	Phenanthrene	Miss.	178.0	0	0.000 µg/l
39	32.644	Anthracene	Miss.	178.0	0	0.000 µg/l
40	35.789	Dibutyl phthalate	Fail	148.9	756923	0.322 µg/l
41	37.898	Fluoranthene	Miss.	202.0	0	0.000 µg/l
42	38.865	Pyrene	Miss.	202.0	0	0.000 µg/l
43	42.717	Benzyl butyl phthalate	Miss.	148.9	0	0.000 µg/l
44	44.515	Benz[a]anthracene	Miss.	228.1	0	0.000 µg/l
45	44.662	Chrysene	Miss.	228.1	0	0.000 µg/l

Fig. 3 GC/Ms-Ms Chromatogram to detect DBP conc

DBP exposure causes a significant reduction in male T, FT, BT, FAI, and T/E2 ratio and significantly elevates E2 level that may be due to anti-androgenic activity of DBP resulting in decreased testosterone production due to interference with steroidogenesis, down-regulating gene and/or protein expression essential for the steroidogenic pathway, and cholesterol transport and metabolism [24,25]. Our findings are consistent with **Radke *et al.*** [17] evidence of an association between DBP exposure and reduction testosterone biosynthesis with elevation of E2 level which disrupts the androgen: estrogen ratio in humans.

Based on previous studies, DBP is the anti-androgenic and anti-estrogenic xenobiotic, which means that they can bind to AR and ER to block the effect of androgens and estrogens on particular cells. Anti-androgens and anti-estrogens block the conformational change of the NR in the complex with the ligand. The NR (nuclear receptors) is unable to obtain active conformation and dissociate chaperones from this complex, which prevents transcription initiation [26, 13, 9] .

Other studies reported that DBP may cause oxidative damage and induced the formation of reactive oxygen species (ROS) in target orang which reason of cells damage and dysfunction [27,28]. Also, exposure to DBP led to disrupting the growth of Sertoli cells and Leydig cells. The Sertoli cells express functional receptors for FSH, whereas the Leydig cells express LH receptors [29].

The reproductive system of males and females are at higher risk of exposure to these chemicals. Unfortunately, it was detected by high dose in studied samples blood. These chemicals accumulated at adipose tissue in our bodies and this continuous exposure could cause many further harmful effects on general health, especially sex hormone and its target organs.

Conclusion

The increased use of phthalates and other EDCs in the plastic products industry in the last period can explain the worldwide higher prevalence of reproductive disorders**.**

The results of current study showed that the detected dibutyl phthalate (DBP) level in the Egyptian adult's blood was significantly associated with many reproductive hormones disorders and many other complications. SO, it is recommended that reduce human exposure to DPB in the food introduce in plastic ware. Also, public awareness of these harmful effects should be strengthened, and protection against DBP exposure should be promoted. However, our findings need to be confirmed by other future studies.

Abbreviations

Alb: Albumin; **EDC:** Endocrine-disrupting chemical; **GC/MsMs:** Gas chromatography–mass spectrometry; **AR:** Androgen receptor; **ER:** Estrogen receptor; **T:** Total testosterone; **FT:** Free testosterone; **LH:** Luteinizing Hormone; **FSH:** Follicle – Stimulating Hormone; **PRL:** Prolactin; **E2:** Estradiol; **SHBG:** Sex hormone-binding globulin; **TSH:** Thyroid stimulating hormone; **ELISA:** Enzyme-linked immunosorbent assay; **FAI:** Free Androgen Index; **BT:** bioactive testosterone; **T/E2 Ratio**: Testosterone / Estradiol ratio; **SPE:** solid-phase extraction.

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