



Association of Phthalate Exposure with Endometriosis and Idiopathic Infertility in Egyptian Women

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Abstract

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BACKGROUND: Phthalates are compounds found in medical supplies, cellophane wraps, beverage containers, metal can linings, and other products. They have the potential to be significant endocrine disruptors. In experimental animals, thereby affecting their reproductive capacity. Endometriosis is a gynecological condition defined by ectopic endometrial glands and stromal development. Exposure to phthalates has been linked to the development of endometriosis in numerous studies. The dangers of phthalates to women's reproductive health and fertility have been widely reported.

AIM: So far, the relationship between phthalates and infertility is not proven so we decided to see if there was a link between the urine phthalate metabolite levels and endometriosis or idiopathic infertility in Egyptian women.

METHODS: Our research was carried out at the infertility outpatient clinic of the Faculty of Medicine of Cairo University. It included 100 female subjects aged 18–40-years-old. Group A (idiopathic infertility; n = 40), Group B (endometriosis; n = 40), and Group C (control; n = 20) were the three age-matched groups that were studied. Using high-performance liquid chromatography (HPLC), the urine levels of mono-2-ethylhexyl phthalate (MEHP) were quantified.

RESULTS: The comparison between the study groups has revealed statistically significant differences regarding the urine MEHP levels between Groups A and B. An analysis of the urine MEHP levels in the study Groups A and B has also revealed that the significantly higher urinary MEHP levels are correlated with the use of dietary plastic containers, the use of cosmetics, and the patients' estrogen levels. Moreover, the urinary MEHP levels of Group A were associated with a history of abortions.

CONCLUSIONS: Higher levels of urinary MEHP are positively associated with female reproductive disorders, specifically endometriosis, idiopathic infertility, and abortion.

Introduction

Several phthalates have been discovered as endocrine disruptors and reproductive toxic compounds in both female and male animals, including di-n-butyl phthalate (DBP) and di-2-ethylhexyl phthalate [DEHP] [1]. DEHP and its derivative mono ethyl hexyl phthalate [MEHP] have been shown in investigations to impair ovarian function and diminish antral follicle development [2]. The DEHP is targeting the antral follicles and it has been demonstrated that both MEHP and DEHP can suppress the development of these follicles in rats, by reducing the estradiol synthesis [3]. Interestingly, in a recent human study, greater urine concentrations of the MEHP and the DEHP metabolites were linked to an early pregnancy loss [4].

Endometriosis is an estrogen-dependent chronic gynecological condition in which a tissue that looks like endometrium grows outside of the uterus. Many risk factors might have a role in the

disease's initiation and progression, including endocrine, hereditary, biochemical, environmental, and immunologic variables [5]. These variables may work together to cause endometriosis; however, the estrogen is considered as the major trophic component of the disease, and estrogen exposure has been shown to exert a critical effect on the disease via estrogen receptors (ERs) [6]. This disease affects around 10% of all child bearing women, with the percentage of infertile women reaching 50% [7].

Environmental variables such as phthalate esters can induce endometriosis by generating oxidative stress, disrupting hormonal homeostasis, or modifying immunological responses. More research is needed, however, to evaluate the significance of these connections [8].

Idiopathic infertility is a situation in which a spouse has failed to conceive after a year of trying, and when a thorough investigation has revealed no explanation for the infertility [9]. It concerns 30–40% of infertile couples [10].

Environmental pollutants have been shown in animal experiments to have a detrimental impact on several elements of the female fertility, including ovulation, follicular number, embryo implantation, and meiosis [11].

Herein, we present the results of a preliminary study of ours as the relationship between phthalates and infertility is still under research aiming to see if there's a link between urine phthalate ester levels and endometriosis or idiopathic infertility.

Materials and Methods

Study design and population

The current prospective case-control study was conducted at Cairo University's Faculty of Medicine's Department of Obstetrics and Gynecology's outpatient infertility clinic (Egypt). The study was conducted between March 2021 and June 2021, and it included 100 female subjects who were attending the infertility clinic, and whose age ranged between 18- and 40 years old. Three age-matched groups were formed: Group A (endometriosis; n = 40), Group B (idiopathic infertility; n = 40), and Group C (control; n = 20). Females aged <18 and >40, with other known causes of infertility (namely, a male factor, tubal disorders, polycystic ovaries, pelvic inflammatory diseases, fibroids) or a malignancy were excluded from the study. The Ethics Committee of the Faculty of Medicine of Cairo University has approved the research protocol of this study (IRB no.MD-229-2019). All participants in the study gave their informed consent.

Study measurements

Information on the general, obstetric, and gynecological details were collected. These included sociodemographic details, such as age, residence, work, smoking habits, and social class (according to educational level and general appearance).

A questionnaire exploring the potential exposure to phthalate sources was also filled, including dietary, environmental, occupational, and medical exposure. The exposure index (EI) was calculated based on the following formula:

$$EI = \text{daily intake (once, twice, thrice)} \times \text{duration of exposure (in years)}$$

Moreover, the hormonal profile of all participants was measured, including the levels of the follicular stimulating hormone (FSH), the luteinizing hormone (LH), prolactin, estrogen, and the thyroid stimulating hormone (TSH).

Standard solutions and sample preparation

The urine MEHP levels were detected. Urine specimens were collected in clean glass containers with the subject coding number written on them. Samples that were turbid or included blood were ruled out. All specimens were kept at -20°C until being tested using high-performance liquid chromatography at Cairo University's Department of Forensic Medicine and Clinical Toxicology. To prevent contamination, no plastic tools were utilized in any sampling or in any experimental operations as part of this work. Chromic acid was used to clean all glass equipment, which were then rinsed with deionized water and methanol. Finally, In 10 mL of acetonitrile, 1 mL of the analytical standard was added to produce the stock solution (containing 1 mg of MEHP in 1 mL).

Chemicals used

MEHP (>97%) was obtained from Sigma-Aldrich, the internal standard benzyl benzoate (>99%) was obtained from Lobachemie, the formic acid ($\geq 99\%$) was obtained from Chem Lab, and the phosphate buffer was obtained from Biodiagnostic, while Carlo Erba group, Inc. provided the ethyl acetate, acetonitrile, and methanol. All of the solvents used were HPLC grade. A direct-Q gradient 8 UV system was used to purify the water (Millipore).

Instruments and chromatographic conditions

HPLC: a Dionex UltiMate 3000 UHPLC, a DIODE ARRAY detector, an auto-sampler, an RS pump, and a column compartment were utilized in our high-pressure isocratic system. A reversed phase (150 mm, 4.6 mm) Hypersil BDS chromatographic column [C18 5- μm particle size] and a solid phase extraction HyperSep glass (with 16 port vacuum manifolds) Rocker 400 Thermo Scientific vacuum pump were used. Thermo Scientific also supplied the SPE columns used in our analysis [HYPERSEP C18500MG/3ML/50PKG].

Methodology

Pretreatment of samples (solid phase extraction): Samples of urine were cooled and homogeneously vortexed. A glass tube was used to hold each 950- μL urine sample, 50 μL of sodium hydroxide were added, and the sample was boiled to 100°C for 1 h (alkaline hydrolysis).

Solid phase extraction: Conditioning: In that order, 1 mL of phosphate buffer solution (pH 2.0), 1 mL of methanol, 1 mL of acetonitrile, and 1 mL of phosphate buffer solution (pH 2.0) were included. Loading: 1 mL of urine was diluted with 1 mL of phosphate buffer solution

(pH 2.0) and then added to the SPE column (with 10 μ L benzyl benzoate). The cartridges were then washed in a solution containing 2 mL formic acid and 1 mL water. Following that, negative pressure was used to dry the cartridges. Elution: 1 mL ethyl acetate and 1 mL acetonitrile were used for elution. The eluent was concentrated and evaporated after it was collected. The 1000 μ L of the mobile phase were used to rehydrate the dry residue.

Chromatographic conditions (mobile phase): 1.0 mL acetic acid was added to 100 mL of HPLC grade acetonitrile to make the 1 L of the mobile phase. This solution was kept in an amber container at ambient temperature. The column was heated to a temperature of 40°C. The injection volume of the sample was 20 L, and the rate of flow was 0.3 mL/min. UV was adjusted between 240 and 280 nm, with a maximum absorption of 254 nm.

Statistical methods

To code and analyze the data, the statistical software for the social sciences (SPSS; version 26) was employed (IBM Corp., Armonk, NY, USA). The mean, SD, median, minimum, and maximum were used to represent quantitative data, while the categorical data were summarized using frequency (count) and relative frequency (%). Quantitative variables were compared using the non-parametric Kruskal–Wallis and Mann–Whitney tests [12]. When comparing categorical data, the Chi-squared test was employed. The exact test was used instead when the expected frequency was <5 [13]. To calculate the correlations between quantitative variables, the Spearman correlation coefficient was utilized [14]. $p < 0.05$ was considered statistically significant.

Results and Discussion

Results

The difference between groups was statistically significant ($p < 0.001$) in terms of mono-2-ethylhexyl phthalate [MEHP] levels in urine samples, as indicated in Table 1. Table 2 presents the association between dietary and environmental exposure and urine MEHP levels, where statistically, there was a difference concerning the correlation of the exposure to dietary plastic containers with urine MEHP levels ($p = 0.007$) and with cosmetics ($p = 0.002$).

The exposure index (EI) and MEHP levels in the urine had a substantial positive correlation ($p = 0.024$) with a correlation coefficient of 0.226

Table 1: MEHP distribution among study groups

	Idiopathic infertility (A)					Endometriosis (B)					Control (C)					p value
	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum	
MEHP in urine (μ g/ml)	136.45	152.88	72.72	1.78	472.30	191.39	126.36	215.61	1.15	420.75	20.92	33.34	5.41	1.85	139.88	<0.001

Kruskal–Wallis and Mann–Whitney tests. Value represented in mean, median \pm standard deviation (SD), $p \leq 0.05$ was considered significant, $p < 0.01$ was considered highly statistically significant.

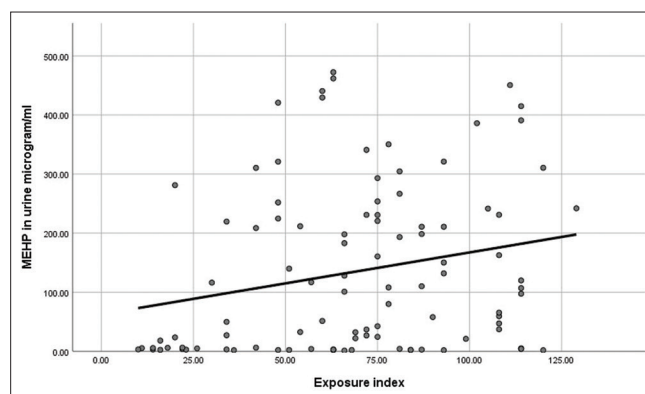


Figure 1: Correlation between exposure index and MEHP in urine

(Figure 1). Estrogen and MEHP levels in urine have shown a statistically significant association ($p < 0.001$) with a correlation coefficient of -0.648 (Figure 2), but LH, FSH, TSH, and prolactin had no statistically significant relationships (Table 3).

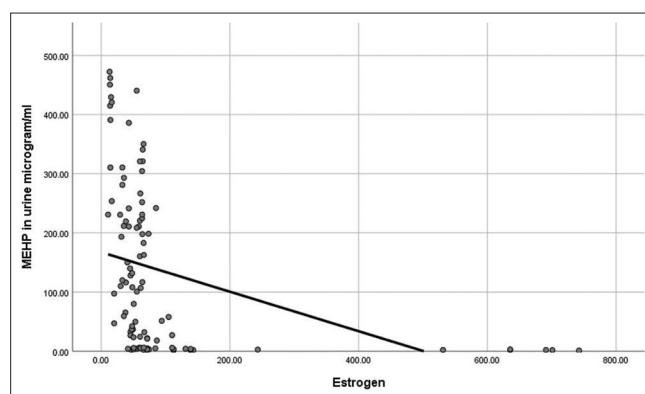


Figure 2: Correlation between estrogen and MEHP in urine

The study Group A has demonstrated a high statistically significant association between the history of an abortion and urine MEHP levels ($p = 0.005$), with a mean value of $233.14 \pm 174.19 \mu$ g/mL, and a median of 220.85μ g/mL (Figure 3).

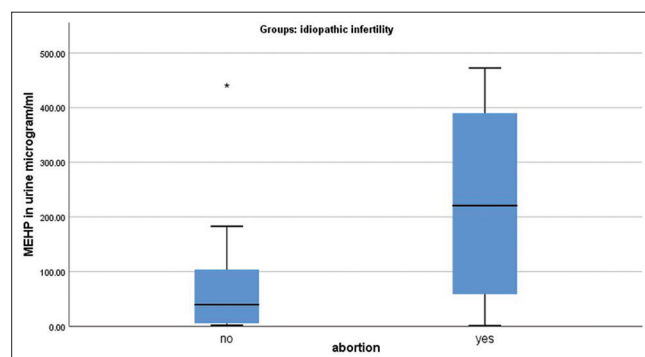


Figure 3: Relationship between abortion and MEHP in study Group A

On the other hand, the usage of dietary plastic containers and urine MEHP levels was shown to be

Table 2: Relation between dietary and environmental exposure and MEHP in urine

Dietary and environmental exposure	MEHP in urine microgram/ml					p value
	Mean	SD	Median	Minimum	Maximum	
Plastic dietary container						
Yes	157.77	143.76	123.89	1.73	472.30	0.007
No	71.44	107.85	6.04	1.15	310.37	
House hold cleaner						
Yes	140.77	140.36	107.53	1.15	472.30	0.054
No	4.67	1.11	5.02	3.08	5.56	
Cosmetics						
Yes	150.90	141.46	116.53	1.15	472.30	0.002
No	39.65	84.56	5.02	2.06	251.90	

Kruskal–Wallis and Mann–Whitney tests, Value represented in mean, median \pm standard deviation (SD), $p \leq 0.05$ was considered significant, $p < 0.01$ was considered highly statistically significant.

statistically significant in the study Group B ($p = 0.026$), but there was no significant association between the use of household cleaners and urine MEHP levels ($p = 0.079$).

Table 3: Correlation between (LH, FSH, TSH, Estrogen, and Prolactin) and MEHP in urine

Hormonal profile	MEHP in urine microgram/ml		
	Correlation coefficient	p value	n
LH	-0.095	0.349	100
FSH	0.044	0.663	100
Prolactin	0.155	0.123	100
Estrogen	-0.648	<0.001	100
TSH	0.087	0.390	100

Spearman correlation coefficient, $p \leq 0.05$ was considered significant, $p < 0.01$ was considered highly statistically significant.

Moreover, our study has identified a significant association between the use of cosmetics and the urine MEHP levels ($p = 0.041$), as shown in Table 4. Finally, Figure 4 demonstrates the statistically significant correlation between the urine estrogen and MEHP levels in study Group B ($p < 0.001$), with a correlation coefficient of -0.659 .

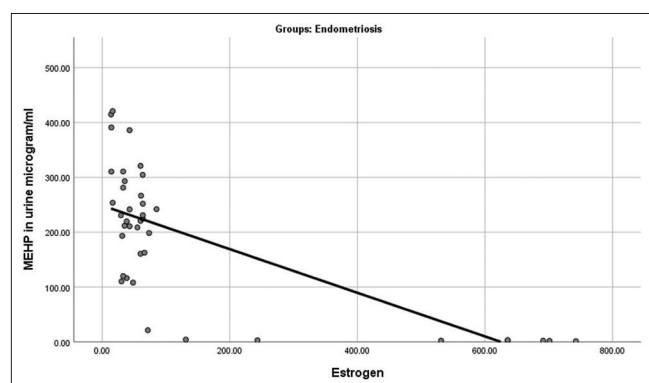


Figure 4: Correlation between estrogen and MEHP in urine in study Group B

Discussion

Phthalates are toxic compounds that are employed in a wide range of industries, including processing and packaging, necessities, medicinal coatings, construction materials, cosmetics, and care products. Phthalates exist in all these objects, and they

may readily poison the environment and cause health issues [15].

Some studies have suggested a link between phthalate exposure and endometriosis, since Estrogens are recognized to be important in the development of endometriosis [16].

Table 4: Relationship between dietary and environmental exposure and MEHP in urine in study group B

Dietary and environmental exposure	Endometriosis (B)					p value
	MEHP in urine microgram/ml					
	Mean	SD	Median	Minimum	Maximum	
Plastic dietary container						
Yes	157.77	143.76	125.72	1.73	472.30	0.026
No	71.44	107.85	5.04	1.15	310.37	
House hold cleaner						
Yes	150.43	158.67	127.88	2.08	414.85	0.079
No	133.83	139.01	97.36	1.15	472.30	
Cosmetics						
Yes	140.90	131.46	113.53	1.15	472.30	0.041
No	29.65	74.56	4.02	2.06	251.90	

Kruskal–Wallis and Mann–Whitney tests, Value represented in mean, median \pm standard deviation (SD), $p \leq 0.05$ was considered significant, $p < 0.01$ was considered highly statistically significant.

With regard to the urine MEHP levels, there was a significant difference between the endometriosis and control groups ($p < 0.001$). The latter can be explained by the findings of a previous study, in which *in vitro* DEHP boosted matrix metalloproteinase-2 and-9 activities, p21-activated kinase-4 expression, and extracellular signal-regulated kinase activation in endometrial cells, these molecules lead to the establishment and progression of endometriosis by enhanced cellular viability and invasiveness in endometrial cells. Endometrial implant development was also found to be faster in DEHP-fed rats (as compared to normal diet fed rats) in the same study. These data clearly imply that an exposure to phthalates can cause endometriosis by increasing the endometrial cells' invasiveness and proliferation capabilities [17].

Phthalates have also been linked to endometriosis due to their ability to cause oxidative stress. In fact, exposure to phthalates raises the generation of reactive oxygen species, while lowering antioxidant enzyme expression (including those of superoxide dismutase and glutathione peroxidase) [18]. Moreover, phthalates have a favorable, dose-dependent influence on the expression of estrogen receptors (ERs) [19]. The activity of phthalates on ERs can also contribute to the development of estrogen-dependent malignancies, including ovarian and breast cancers [20].

Our findings are consistent with those of the previous studies [21], [22]. However, our findings are not in agreement with another previous study that has not discovered a significant connection between DEHP levels and endometriosis ($p = 0.161$), but a statistically significant weak association between MEHP levels and endometriosis ($p = 0.020$) in their case–control study [23].

Other researchers have revealed that the concentrations of the phthalate byproducts di-n-butyl phthalate (MBP) and MEHP in endometriosis patients

are not significantly higher ($p = 0.1$) than those in controls [24]. A meta-analysis has found that only the MEHP levels can be potentially associated with endometriosis [25]. This inconsistency can be explained by the fact that different countries have different levels of Phthalate exposure, which plays a big part, and different races have different results. Weight difference, sample size difference, age variance, health state, concentration and length of exposure of study subjects, or different detection technologies, all contribute to heterogeneity.

With regard to the urine MEHP levels measured in our study, there was an important distinction between Group A and the control group ($p = 0.007$). The latter can be explained by high levels of MEHP reducing ovarian proliferative capacity in a dose-dependent manner and altering gene expression in developed oocytes [26].

Furthermore, *in vivo* studies have demonstrated that the antenatal and the postpartum exposure to DEHP can change the number of dominant follicles [27]. Recent research has found that phthalate exposure during pregnancy and after delivery may have harmful consequences for women [28]. Moreover, higher urine MEHP levels have been related to a decreased likelihood of conception and live birth in *in vitro* fertilization (IVF) patients; in fact, the findings show that increased phthalate toxicity in spouses may impair the undertaken IVF [29].

Phthalates impact oocyte meiotic maturity and ovulation by changing hormone production and by raising reactive oxygen species in the developing follicles. Their detrimental impact on oocytes is mediated by the surrounding somatic cells' overactivity of estradiol-dependent epigenetic pathways. DEHP can cause epigenetic alterations in later generations due to the fact that the ER- α can interact with numerous enzymes that are responsible for epigenetic changes [30].

Our results do not agree with recent study, in which there was no significant relationship identified between DEHP metabolites and any clinical outcomes [31].

Sifakis *et al.* have found that, despite the high urine phthalate values (particularly those of MEHP being around 0.69 g/L) being linked to a greater risk of IVF patients' implantation failure [11].

In the current study, a significant correlation was found between the use of dietary plastic containers and the urine MEHP levels. This could be due to the fact that phthalate migration levels were higher when plastic containers were used for longer periods of time and heating times were longer [32], while significant DEHP and DBP levels were discovered in plastic dinnerware at room temperature [33]. Both phthalates and bisphenol A can be found in plastics used for food storage and cooking utensils [34].

Endocrine disruptor chemicals can leach into food and beverages from plastic containers and plates,

especially when it becomes hot or cold. Data suggest that the latter is significant human exposure sources to endocrine disruptor chemicals [35].

There were no significant differences between working in a plastic manufacturing industry or using household cleaners and the urine MEHP levels; this can be explained by the low number of women working in the plastic manufacturing industry in our study ($n = 3$; mean MEHP levels: 150.43 $\mu\text{g/mL}$). This finding is also not in agreement with that of a study conducted by Pan *et al.*, [36].

As women are primarily responsible for domestic tasks, they may be exposed to greater quantities of phthalates through the use of chemicals [37]. Furthermore, due to the fact that phthalates are commonly used as stabilizing agents in aesthetic goods, skin adsorption of these compounds is possible [38].

The present study has revealed a significant correlation between the use of cosmetics and the urine MEHP levels. Moreover, many participants in this study were heavy users of cosmetics. Furthermore, the bulk of the participants in our study were classified as belonging to a low or moderate social class. As a result, the majority of the participants this group may utilize low-quality, low-cost cosmetics that contain high levels of phthalate. This is consistent with Darvishmotevalli *et al.* findings, which found a strong link between personal care product use and urine MEHP ($r = 0.48$) or MEHHP ($r = 0.55$) levels [39]. On the other hand, studies have revealed no link between skin care product use and phthalate metabolite concentrations in urine samples which can be explained by different population, sociodemographic and lifestyle characteristics [40].

There were considerable variances between our study groups in terms of the LH and TSH levels. This can be explained by fact that the control of the hypothalamic pituitary gonadal axis is disrupted by phthalates. The GnRH, LH, and FSH levels are all affected; as a result, the action of steroidogenic enzymes is disrupted, thereby causing a knock-on impact on sex hormones [41].

Furthermore, in a research by Liu *et al.*, the blood levels of FSH, LH, and testosterone were found to be substantially lower in the rats given 500 and 1000 mg/kg/d of DEHP (as compared to the control rats) [42]. In a study conducted by Gao *et al.*, no significant relationships between MEHP and TSH were identified [43].

MEHP and MEHHP have been previously shown to be favorably linked to TSH [44], whereas MEHP and mono isobutyl phthalate have been found to be inversely associated with TSH [45]. While MEHP has been shown to be positively associated with FSH and LH by Al-Saleh *et al.*, phthalate exposure is also linked to altered reproductive hormone levels and semen-associated parameters [46].

Phthalates exert an anti-androgenic action by lowering testosterone levels while also lowering estrogen levels in large doses [47]. Mature mice receiving a high DEHP dosage (2000 mg/kg/day) have been shown to have longer ovulatory cycles, lower blood estrogen levels, and no ovulation [48]. This can explain the negative correlation between estrogen and MEHP levels that have been found in the present study ($p < 0.001$) with regard to Groups A and B. Similarly, Cao *et al.* have examined the urinary levels of phthalate metabolites as well as the serum levels of ovarian hormones, and have discovered that the estrogen to FSH ratio (an indicator of the ovarian reserve) was significantly and adversely linked with urine levels of most phthalate end products in control women [49].

Our findings are also consistent with those of Fu *et al.*, who found that a 30-day continuous exposure to DEHP in mice reduced the ovarian organ coefficient and drastically reduced estrogen levels [50].

These studies can support the association between phthalates and idiopathic infertility and it might suggest the existence of another mechanism by which the MEHP can induce endometriosis (without involving the estrogenic theory).

In polyvinyl chloride workers, a significantly positive relationship between the urine concentrations of DEHP metabolites and estrogen, as well as between the proportion of estradiol to testosterone, has been identified [51]. This study attributes the link between MEHP and endometriosis to the estrogenic properties of MEHP.

In the present study, there was a significant association between the history of an abortion and the urine MEHP levels in Group A ($p = 0.005$; mean MEHP levels: $233.14 \pm 174.19 \mu\text{g/mL}$). This can be explained by the fact that phthalates have an impact on the duration and progress of gestation. A DEHP exposure during pregnancy in rats at 250 and 500 mg/kg [52] and at 50 and 200 mg/kg [53] has been shown to impair the placental vasculature and to cause a miscarriage and an obstructed labor in subsequent generations. This finding is consistent with that of the case control study conducted by Yi *et al.*, who found that the urinary levels of metabolites of di-(2-ethylhexyl) phthalate (DEHP) and dimethyl phthalate (DMP) were significantly higher in the cases (150 missed miscarriage) than in the controls (150 normal pregnancies). A strong dose-response relationship was observed between urinary metabolite levels and the odds of missed miscarriage [54].

The MEHP levels were found to be substantially higher in women who had miscarriages in this study. This can be explained by the association between MEHP and peroxisome proliferator activated receptors [PPARs] or prostaglandins-related miscarriages as a possible mechanism, both of which being known to cause pregnancy disorders (namely, extended pregnancy, shortening of pregnancy, and

miscarriage) [41]. Both the PPARs and prostaglandins can be modulated by phthalate metabolites (such as DEHP and MEHP). PPARs are required for the continuation of a gestation. DEHP and its derivatives can bind to PPARs, thereby preventing the maternal-fetal connection required in order to start labor [55]. On the other hand, prostaglandins are signaling chemicals that cause the uterus to contract, thereby resulting in delivery or miscarriage. DEHP is known to induce prostaglandin production, which, in turn, can lead to a pregnancy loss or a premature labor [56].

Conclusions

Our study identified a statistically significant correlation between female reproductive disorders (specifically, endometriosis, and idiopathic infertility) and the levels of mono-2-ethylhexyl phthalate [MEHP] in urine. Moreover, significant correlations between social class, use of dietary plastic containers, use of cosmetics (containing phthalate), history of abortions, and estrogens level were identified. The urine of exposed cases had a significantly higher level of MEHP.

Authors' Contributions

This article was prepared with equal contributions from all authors.

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