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Evidence About the Possible Role of Phthalates and Bisphenol A in Recurrent Pregnancy Loss and Endocrine Dysfunctions: A Case–Control Study

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Abstract: Objectives. A case–control study was conducted to investigate the exposure levels to some specific chemicals, in women with infertility issues, compared with fertile women. Methods. A total of 186 cases and 196 controls were recruited. Each participant provided a urine sample for the determination of six phthalate metabolites (mono-ethyl phthalate, MEP; mono-n-butyl phthalate, MnBP; mono-n-ottyl phthalate, MnOP; monobenzyl phthalate, MBzP; and two metabolites of the diethyl-hexyl phthalate (DEHP): mono(2-ethyl-5-hydroxyhexyl) phthalate, MEHHP and mono(2-ethylhexyl) phthalate, MEHP) in addition to bisphenol A, BPA. Each woman also completed a questionnaire. The urine samples were analyzed using HPLC/MS/MS methods. Results. The analysis revealed significantly higher metabolite concentrations in cases than in controls for all metabolites, except MnOP. Stratification based on infertility factors, showed a significant association of MnBP, MBzP, BPA and DEHP with ovulatory and endocrine dysfunctions. Furthermore, higher mean concentrations of MEP and DEHP were observed in women with recurrent pregnancy loss (RPL) and idiopathic infertility, respectively. Conclusion. These findings suggest that some of the analyzed chemicals may play a role in female infertility. Exposure to DEP (diethyl phthalate) and DEHP appears to be associated with RPL and idiopathic infertility. Further investigation is required to explore potential sources of these risks.

Keywords: phthalates; reproduction; women; BPA; endocrine disrupters

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1. Introduction

Infertility is defined as the inability to conceive after 12 months, or more, of unprotected sexual intercourse [1]. It has a multifactorial etiology, influenced by a range of risk factors that may affect female reproductive capacity. This risk factor can arise from both personal and occupational exposures [2]. In recent years, increasing attention has been focused on xenobiotics classified as endocrine disruptors, which interact with estrogen and androgen receptors [3–5]. Historically, these chemicals have been widely

used [6], notably as additives in plastic materials, solvents and stabilizing agents in cosmetics. They were also present in thermal papers and as coating agents in food cans. Currently, numerous legal restrictions exist, particularly in Europe, to reduce this type of risk.

Phthalates represent a class of structurally similar molecules that have been used industrially since the early 20th century. Their reprotoxicity is well documented in the literature [7]. In vivo studies have demonstrated embryotoxicity and fetotoxicity [8,9] in chronically exposed female mice. In human studies, long-term exposure to phthalates has yielded conflicting results regarding their role in pregnancy loss [10–12]. Moreover, other studies have identified associations between phthalate exposure and reduced follicle numbers [13], as well as a decreased number of retrieved, mature and fertilized oocytes [14,15]. Further investigations have highlighted the potential reproductive toxicity of di-(2-ethylhexyl) phthalate (DEHP), diethyl phthalate (DEP), dibenzyl phthalate (DBzP), di-n-butyl phthalate (DnBP), butylbenzyl phthalate (BBzP), and more recently di-n-octyl phthalate (DnOP) and di-iso-nonyl phthalate (DiNP) [16].

Bisphenol A (BPA), structurally similar to 17-β-estradiol, can bind to estrogen receptors (α and β) [17–19]. Both in vivo and in vitro experimental studies have emphasized the reprotoxic effects of BPA, which result in oocyte aneuploidy [20], chromosome segregation [21] and effects on meiotic segregation [22]. However, results from human studies remain inconclusive. While some studies found BPA levels to be negatively correlated with estradiol levels [23,24] and reduced useful oocyte numbers in assisted reproductive technology (ART) cycles [25], other studies have yielded contradictory findings [26]. Furthermore, BPA has also been shown to exhibit antiandrogenic activity [27]. In vitro and in silico assays have demonstrated that BPA significantly antagonizes the androgen receptor (AR) by 5α-dihydrotestosterone (DHT)-induced AR transcriptional activity [27].

Exposure to these chemicals occurs primarily through inhalation, dermal absorption and ingestion [28,29], particularly via food contamination [30,31]. Phthalates are rapidly metabolized into monoesters and oxidative metabolites [32], which are excreted predominantly in urine. For this reason, measuring urinary metabolites of phthalates and BPA is considered the most reliable method to assess environmental exposure, and such molecules are useful as biomarkers. Complete urinary excretion occurs within 24 h, with a peak in urinary levels approximately 4 h after exposure [33,34].

Given the potential reproductive toxicity of these substances, studying infertile women may provide insights into their heightened vulnerability [35]. Therefore, identifying circumstances under which exposure occurs, whether in the workplace or daily life, could help to clarify the real routes of exposure and promote reproductive health.

The present study was conducted to assess if exposure to phthalates and BPA could be linked with reproductive problems in a population of women with diagnosis of infertility, and to explore possible ways of exposure. The aim of this study is to understand whether the infertile population actually shows, as suggested in the literature, higher exposure values for these substances and whether these exposure levels can be correlated with specific infertility factors.

2. Materials and Methods

2.1. Study Population

This epidemiological study enrolled female users of a fertility center as cases. Inclusion criteria were as follows: age under 43 years, no history of chemotherapy related to genital problems, and no infertility issues related to surgery or anatomical alterations. The minimum age of the sample of infertile women was 34 years, as younger women were

not present at the assisted reproduction center during the sampling period. It should be noted that the average age of the first pregnancy in Italy is estimated at 32 years, which may contribute to the likelihood of noticing reproductive issues at an older age.

Urine samples were taken from non-pregnant women diagnosed with infertility at the clinic. In this survey, women were explicitly selected from couples where infertility was exclusively attributed to the female partner, while the semen quality of their partners was analyzed.

Confounding factors considered in the statistical elaboration, prioritized in relation to the studied situation, included body mass index (BMI), smoking habits, alcohol consumption, age between 35 and 43 years, prior chemotherapy treatments (excluding genital treatment), insulin-dependent diabetes [36] and thyroid disorders [37]. A final exclusion criterion was an abnormal urinary creatinine level, based on World Health Organization (WHO) guidelines [38].

The calculation of the sample size useful for the significance of the data was carried out starting from the prevalence of the considered pathology: the prevalence data of female infertility in Italy is equal to 15% [39], and a margin of maximum error of 5% and a confidence level of 95% was set.

After applying all criteria, a total sample of 186 eligible women with infertility issues was identified.

The control group consisted of women who had recently given birth in the same hospital's obstetrics unit, without undergoing hormonal or fertility treatment, and who achieved pregnancy within 12 months. Control samples were collected at least 4 days postpartum, resulting in an overall control sample of 196 female subjects.

The Institutional Review Board of the IRCCS San Raffaele Scientific Institute in Milan approved the investigation protocol, assigning it the identification code 73/INT/2017. All procedures involving human participants were conducted in accordance with the Declaration of Helsinki.

All participants signed an informed consent form and subsequently completed a structured questionnaire to collect data on clinical status, with a focus on endocrine conditions, lifestyles and occupational habits. The questionnaire adopted involved a multidisciplinary team in its drafting, made up of gynecologists expert in assisted reproduction, embryologists, statisticians and experts in chemical risk in living and working environments.

A well-trained researcher assisted in the completion of the clinical anamnesis. The questionnaire's life habits section collected information on smoking, alcohol consumption, the use of plastic containers, the use of cosmetics such as scents, hair sprays and nail polish, and dietary habits.

Every participant provided a random spot urine sample during her morning clinical visit. These urine samples were analyzed for chemicals of interest.

The sampling period extended from October 2017 to June 2019, covering the geographic area of Northen Italy.

2.2. Analytical Procedure

The analytical phase employed previously published HPLC tandem mass spectrometry methods [40,41]. Briefly, for phthalates analysis, urine samples underwent a pretreatment involving enzymatic digestion with β-glucuronidase-from E. Coli, followed by solid-phase extraction (SPE OASIS HBL, 6 cm^3, 200 mg cartridges) before the chromatographic column injection. For BPA analysis, the samples were first incubated at 38 °C for 2 h with β-glucuronidase-arylsulfatase enzyme from Elix Pomatia, followed by acidification with acetic acid 2% (*v*/*v*). The analyte was then extracted using SPE extraction with SPE OASIS HBL (6 cm^3, 200 mg) cartridges, before the HPLC-MS/MS analysis.

The main metabolites of phthalates of interest were analyzed: in detail, mono-ethyl phthalate (MEP) from DEP; mono-n-butyl phthalate (MnBP) from DnBP and BBzP; mono-n-ottyl phthalate (MnOP) from DnOP; monobenzyl phthalate (MBzP) from BBzP and DBzP; two metabolites of the DEHP: mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and mono(2-ethylhexyl) phthalate (MEHP); and total BPA.

Urinary levels were normalized by dividing by urinary creatinine, obtained using the Jaffè's method [42].

Samples with creatinine levels exceeding 3 g/L or below 0.3 g/L were rejected, following the WHO guidelines [38] and the American Conference of Governmental Industrial Hygienists (ACGIH) recommendation [43]. Values outside this range could indicate kidney issues or other diseases, leading to erroneous results.

2.3. Statistical Elaboration

The SPSS® 25.0 (IBM, Armonk, NY, USA SPSS) software was used for statistical analysis. A preliminary descriptive analysis was conducted to assess the characteristics of the sample population and the distribution of urinary metabolite concentrations. For results below the limit of detection (LOD), values were established to LOD divided by two [44]. Non-parametric methods were employed to analyze the phthalate metabolite concentrations, to discriminate the differences between different groups, including occupational activities, personal habits and demographic categories.

To highlight the differences between cases and controls, the odds ratio was calculated, considering working activities and the daily life habits. Associations between urinary metabolites and infertility factors were assessed using the Kruskal–Wallis test and Dunn post hoc test. The statistical elaboration considered the confounding factors using multivariate logistic regressions.

3. Results

Table 1 shows information about the populations studied, highlighting possible significant differences in specific parameters. The two groups of women display similar characteristics, although infertile women had a higher mean older age and a greater tendency to smoke. Regarding the body mass index, the women controls were asked to indicate their weight before the beginning of the pregnancy.

Table 1. Characteristics of population under study.

	Cases (n = 186)	**Controls (n = 196)**	***p* Value**
Age (range)	37.6 (29–43)	33.4 (21–44)	0.000 *
BMI [a] (% of subjects in the class)			
Normal	73.3	59.2	0.101
Overweight	11.3	14.8	
Obese	4.3	8.7	
Underweight	9.1	8.7	
Unknown	1.6	8.7	
Present smokers (%)	16.1	6.1	0.010 *
Previously smokers (%)	23.1	20.4	
Alcohol consumption (%)			
Daily	5.9	7.7	0.000 *
Weekly	44.6	26.0	
Monthly	23.7	28.1	
Never	21.0	37.2	
Missing	4.8	1.0	
Residence area (%)			

Urban	79.0	85.7	0.469
Rural	11.8	9.2	
Coast	2.2	1.0	
Industrial	1.1	0.0	
Urban and industrial	1.1	0.0	
Other	3.7	0.5	
Missing	1.1	1.5	
Use of plastic containers for fat food storage (%)			
Never	17.2	14.3	0.244
Daily	23.1	21.4	
Weekly	38.2	46.9	
Monthly	18.8	16.8	
Missing	2.7	0.5	
Eating canned food at least weekly (%)	43.5	40.3	0.290
Eating soya products at least weekly (%)	17.8	11.8	0.070
Use of scents at least weekly (%)	80.1	80.7	0.560
Use of nail polishes at least weekly (%)	40.9	32.6	0.148
Use of hair sprays at least weekly (%)	16.6	14.3	0.399
Working activity (%)			
Armed forces	0.5	0.0	0.110
Industrial workers	1.1	2.6	
Education/learning area/ professionals/PC operators	60.7	58.2	
Health workers	8.6	11.7	
Cleaning activity/catering	7.5	6.6	
Trade	8.1	5.6	
Unemployed	2.2	4.1	
Others	11.3	11.2	

[a] BMI: body mass index. * Significant difference; Mann–Whitney test for continuous variables; χ^2 test for qualitative variables.

Table 2 presents the results of the urine sample analysis, detailing the number of samples with metabolites exceeding the limit of detection (LOD) of the methods, and the potential differences highlighted using the Mann–Whitney test. Overall, the results showed significantly higher values in infertile women for all the metabolites, with the only exception being for MnOP. Higher exposure was confirmed by the elevated urinary analyte levels in the cases group.

Table 2. Results (μg/g creatinine) of urinary metabolites of phthalates and BPA.

Parameters	**MnBP** [1]		**MEP** [2]		**MBzP** [3]		**MnOP** [4]		**DEHP** [5]		**BPA** [6]	
	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls
N subjects	186	196	186	196	186	196	186	196	186	196	125	112
Arithmetic mean	25.69	21.76	191.82	101.06	5.53	2.75	2.14	1.86	16.52	11.08	1.73	0.90
Median	16.29	11.55	13.26	28.59	2.62	1.33	1.25	1.03	8.57	5.65	0.57	0.24
Standard deviation	38.39	53.24	762.55	292.23	11.47	3.83	2.69	1.86	24.30	11.08	3.82	4.16
5° percentile	1.25	1.25	0.77	1.50	0.27	0.38	0.08	0.29	1.15	0.92	0.05	0.07
95° percentile	108.39	58.02	635.08	421.62	23.72	9.71	8.44	6.18	58.26	28.52	8.49	2.88
p value (Mann–Whitney test)	0.011 *		0.036 *		0.000 *		0.526		0.000 *		0.000 *	
LOD [7] (μg/L)	0.8		3.0		1.2		0.1		0.15 MEHP [8] 0.05 MEHHP [9]		0.02	
% > LOD	97.3	97.4	62.9	79.1	69.4	51.5	91.4	100.0	99.5	100.0	99.2	100.0

[1] MnBP—mono-n-butyl phthalate; [2] MEP—mono ethyl phthalate; [3] MBzP—monobenzyl phthalate; [4] MnOP—mono-n-octyl phthalate; [5] DEHP—di-(2-ethylhexyl) phthalate, molar sum of two

metabolites; [6] BPA—bisphenol A; [7] LOD—limit of detection; [8] MEHP—mono(2-ethylhexyl) phthalate; [9] MEHHP—mono(2-ethyl-5-hydroxyhexyl) phthalate. * Significant value.

MEP levels, despite showing considerable variability, were consistently higher than the concentrations of other phthalates. This may be because DEP is the only compound considered here that is not subject to legal restrictions in Europe [45]. The exposure values were also one or two orders of magnitude higher, with peaks reaching up to 7000 μg/L.

Instead, Table 3 stratifies the data according to the infertility factors reported by the infertile women. Differences among the groups were evaluated using the Kruskal–Wallis test, followed by Dunn's post hoc test for a more detailed analysis of the differences between each infertility factor and the controls. Statistically significant differences, between cases and controls, in urinary levels of MEP MnBP, BPA and DEHP were observed in women with infertility related to ovulatory factors or endocrine dysfunction. MEP levels also differed significantly from controls in women with recurrent pregnancy loss and reduced ovarian reserve. Additionally, DEHP levels were significantly elevated in women with ovulatory, endocrine and idiopathic infertility compared to controls.

DEHP showed higher mean concentration levels, compared with mean value in controls, in all groups of the different infertility factors, except for reduced ovarian reserve, with statistical significance in ovulatory and endocrine dysfunction and idiopathic infertility groups (sine causa infertility). MnBP levels were twice as high in women with ovulatory and endocrine dysfunction, and the same trend was observed for MBzP.

MEP levels were notably higher in women with RPL, with the geometric mean concentration being twice that of the controls. Finally, BPA levels were statistically significantly elevated in women with ovulatory and endocrine dysfunction, and an increasing trend was observed in women with endometriosis (mean value: 0.94 ± 3.93 μg/g creatinine), tubal factors (1.07 ± 5.43 μg/g creatinine) and reduced ovarian reserve (0.61 ± 2.58 μg/g creatinine) compared with controls (0.28 ± 3.06 μg/g creatinine), although these differences were not statistically significant.

Table 3. Phthalates levels with stratification for infertility factors vs. controls.

		μg/g Creatinine (Geometric Mean ± SD [a])					
Infertility Factors	**N**	**MnBP**	**MEP**	**MBzP**	**MnOP**	**DEHP**	**BPA**
Controls	196	9.32 ± 3.76	22.86 ± 6.45	1.49 ± 2.94	1.11 ± 2.48	5.46 ± 2.76	0.28 ± 3.06
Ovulatory and endocrine dysfunctions	30	21.95 ± 3.84 *	10.71 ± 9.08	3.97 ± 3.80 *	0.81 ± 4.27	12.64 ± 3.06 *	0.64 ± 2.31 *
Endometriosis	20	15.07 ± 2.94	29.04 ± 6.02	2.02 ± 3.33	1.14 ± 3.21	8.78 ± 2.43	0.94 ± 3.93
Idiopathic	52	13.44 ± 2.74	19.49 ± 10.81	2.26 ± 3.48	1.38 ± 4.35	9.69 ± 2.99 *	0.44 ± 4.56
Recurrent Pregnancy Losses (RPL)	18	14.52 ± 4.50	40.07 ± 21.03 *	2.38 ± 2.54	0.56 ± 3.25	10.61 ± 2.56	0.41 ± 3.68
Reduced ovarian reserve	50	8.72 ± 3.66	7.67 ± 7.07 *	1.74 ± 3.48	1.02 ± 3.11	5.39 ± 3.50	0.61 ± 2.58
Reduced ovarian reserve + endometriosis	3	14.70 ± 1.08	10.88 ± 7.19	0.57 ± 1.54	1.30 ± 1.51	6.03 ± 1.81	-
Tubal factors	13	13.82 ± 2.73	12.86 ± 8.87	3.31 ± 5.28	1.64 ± 3.50	11.77 ± 3.84	1.07 ± 5.43
p value Kruskal–Wallis		0.027 *	0.021 *	0.001 *	0.135	0.000 *	0.000 *
p value Dunn post hoc test		0.029 *	0.029 * 0.025 *	0.004 *	0.135	0.019 * 0.007 *	0.016 *

[a] SD—geometric standard deviation; * statistically significant ($p < 0.05$).

Tables 4 and 5 show the calculated odds ratio between cases and controls, considering the different occupational activities and daily life habits, respectively.

No specific occupation or habit seems to be of higher concern in our sample, with the only exception being the use of canned food (OR = 2.021, 95% CI 1.022–3.996).

Table 4. Odds ratio for working activity, adjusted for age, BMI, smoke, alcohol and pathologies.

Working Activity	Cases	Controls	OR	95% CI	*p* Value
Soldiers/policewomen	1	0	-	-	-
Industrial workers	2	5	2.307	0.348–15.293	0.386
Teachers/professionals	112	114	0.699	0.412–1.187	0.185
Nurses/doctors/health professionals	16	23	0.759	0.330–1.747	0.517
Cleaning women/caterer	14	13	2.060	0.721–5.887	0.177
Cashier/traders	15	11	2.580	0.870–7.646	0.087
Other	4	8	0.559	0.107–2.919	0.490

Table 5. Odds ratio for life habits, adjusted for age, BMI, smoke, alcohol and pathologies.

Habits	Cases	Controls	OR	95% CI	*p* Value
Use of plastic containers for storage of fat food	147	167	0.930	0.480–1.804	0.830
Use of canned food	155	154	2.021	1.022–3.996	0.043 *
Use of soya products	90	86	1.111	0.669–1.844	0.685
Use of scents	162	180	0.607	0.265–1.387	0.236
Use of nail polish	150	160	0.634	0.327–1.228	0.176
Use of hair spray	51	49	1.357	0.749–2.459	0.313

* Significant value.

4. Discussion

The results of this study suggest a possible association between exposure to phthalates and BPA and certain female infertility factors. Overall, there was a statistically significant difference between cases and controls, with higher levels of all metabolites in the infertile women, except for MnOP.

Data stratified by infertility factors indicated significantly higher concentrations of MnBP, MBzP, DEHP and BPA in women with ovulatory problems and endocrine dysfunctions. Otherwise, MEP levels were particularly elevated in women with RPL and reduced ovarian reserve, while DEHP levels were higher in women with idiopathic infertility.

The interaction between these chemicals and the endocrine system may directly interfere with endocrine function, potentially leading to ovulatory problems, as shown in experimental studies. These chemicals can affect the quality of the oocyte [46], and recently, some authors [47] have demonstrated a direct interference action of phthalates on the key pathways involved in oocyte maturation.

Experimental data support our findings with the group of women with endocrine and ovulatory problems. Many of the chemicals examined interact with estrogenic or androgen receptors, often involving the aryl hydrocarbon receptor in biochemical mechanisms [48]. BPA can interfere, at different stages, with the feedback control system of the hypothalamic–pituitary–gonadal (HPG) axis, contributing to reproductive toxicity by altering the gonadotropin-releasing hormone levels, which affect follicle-stimulating hormone (FSH) and luteinizing hormone (LH) release [49]. Similar mechanisms are supposed to be involved for phthalates through the HPG axis alteration. Although the involved mechanism remains uncertain, phthalates have been shown to either trigger or inhibit receptor activity, inducing both positive or negative responses on androgen and estrogenic receptors [50]. In vivo studies have demonstrated that phthalate exposure can impair oogenesis [51–53] and folliculogenesis [54], causing damage to deoxyribonucleic acid in

oocytes and altering steroidogenesis and the expression of gonadotropin and hormone receptor signaling [55]. Some studies [16] have suggested that phthalates may contribute to the onset of endometriosis through the action of phthalates on inflammation, cytokine production, oxidative stress increase and proliferation of endometrial cells, though our results did not confirm this hypothesis.

BPA exposure has been associated with a reduced likelihood of embryo implantation and oocyte counts [56,57], as well as other gynecological disorders [58]. BPA tends to bioaccumulate, promoting nongenomic signaling pathways, altering women's metabolism and reproductive function leading to conditions such as hyperandrogenism, insulin resistance, obesity, dyslipidemia, chronic inflammation, anovulation and polycystic ovary syndrome (PCOS) [59]. A case–control study [60] on 321 women with PCOS and 412 controls revealed an increased odds ratio for bisphenol exposure (OR 1.26, 95%, CI 1.12–1.45). Other authors have confirmed the positive association between blood BPA concentrations and PCOS [61]. The current evidence [60,61] suggests a role for BPA, similar to other plasticizers, in female reproductive health, with mean concentrations ranging from 1.00 to 2.70 μg/g of creatinine. This warrants further investigations into the mechanism of action.

Otherwise, epidemiological studies have also highlighted an association between phthalate exposure and infertility, particularly focused on possible effects on oocyte yield [62]; some authors suggested effects like ovarian failure, anovulation and a decrease in steroidogenesis [53]. Though these results are controversial [24].

Regarding the possible association between phthalate exposure and RPL, different studies have focused mainly on DEP, DEHP and DnBP, either individually or in mixtures. However, the evidence remains inconclusive or conflicting [11,63].

Some authors [52] conducted a case–control study focused on RPL (260 patients and 203 controls), finding significantly higher levels of phthalates in cases, especially DEHP (the highest quartile of concentration was strongly related to RPL). The geometric mean of that group of women was 0.27 μg/L (unfortunately, results were presented without creatinine correction), which is markedly lower than the value found in our population (DEHP 10.61 μg/g of creatinine). Messerlian et al. [64] found a similar correlation between DEHP and RPL. Elevated MEP and DEHP levels have been associated with an increased risk of miscarriage [10] in 3220 pregnant women (OR = 1.99 and OR = 2.19, respectively), particularly during early gestation (6-10 weeks of gestation). In later gestation, an association was found only with MEHHP levels (OR = 2.41). However, other authors [12] have not confirmed these findings.

The present study does not highlight a particular risk for DnBP exposure in women with RPL, though previous studies have suggested such an effect [65], observing a solid significance for DnBP exposure and an elevated risk for RPL, with a less consistent correlation with DEHP and DEP metabolite levels [65]. Comparisons with these studies reveal higher DEHP and DEP exposure levels in our sample, up to two orders of magnitude higher, with similar results for DnBP. Conflicting results could be attributed to the differences in study design, exclusion criteria, or the sample size. A meta-analysis considering data on 4713 women (651 cases and 4062 controls) identified an elevated risk of pregnancy loss for MnBP and DEHP (OR = 1.34 and 1.79, respectively), though the authors advised caution due to limited study numbers (only eight) [66]. These data suggest the need to compare findings coming from similar geographic areas to avoid differences due to lifestyle habits and different laws for consumer protection.

Considerations about exposure to mixtures of compounds are mandatory, because even at low doses, different chemicals could determine the same effect. Precisely, in light of these considerations, in a cohort study on 132 women, MEP, MnBP and MiBP levels were associated with a higher risk of RPL [67]. Comparing these results with the data

herein presented a significantly higher geometric mean for MEP (40.07 vs. 13.26 μg/g creatinine).

Epidemiological studies about women undergoing ART are of particular interest for our debate. DEHP confirmed its reprotoxicity, with lower probability of implantation (−22%) and lower possibility of clinical pregnancy (−24%) or the birth of an alive child (−38%) in case of higher concentrations [66]. Higher exposure (to DnBP and DEHP) and exposure to multiple phthalates (DEHP, DiNP, BBzP) led to a significant association with a lower chance of pregnancy [68]. With a female population of 663 subjects, urinary MnBP was negatively correlated with the odds of normal fertilization [15]. Finally, a risk of preterm birth was highlighted in the case of preconception exposure, studying a sample of 386 women, considering DEHP exposure [69].

Our findings strongly suggest a role of DEP exposure in the RPL, endorsing what other authors suggested. Instead, epidemiological investigations carried out to understand the role of phthalates in idiopathic infertility have not been conducted to date, to our knowledge, even though our results showed a possible role of DEHP exposure.

The higher urinary levels of the examined metabolites call for considerations regarding the sources of exposure. The stratification of data in relation to occupational activities was not significant, not allowing us to identify specific workplaces with a higher potential exposure. The difference between cases and controls, however, requires further investigation in daily life habits, as certainly some sources of exposure in the context of life led to higher exposures in the studied infertile women.

Furthermore, data related to DEP exposure are quite interesting, particularly as the DEP is currently not subject to any regulatory restriction in Europe. The mean values of DEP metabolite in cases are twice as high as those of controls, in particular, for women affected by RPL, and at least one order of magnitude higher than the other metabolites.

Limitations

A limitation of this study is the use of random spot urine samples, as these reflect exposure over the 12–24 h preceding sampling. However, as some researchers have observed [70], multiple urinary samples tend to fall within the same quartile of concentrations, possibly due to low variability in the underlined exposure in daily life. In particular, intra-subject variability studies [71–73] have shown that the use of a spot urine sample can be considered moderately representative of exposure to phthalates, with some differences from phthalate to phthalate.

Another potential bias is the possibility that women who had given birth a few days before, a major part of our controls, could have a faster toxicokinetic than other women, and this could, to a certain extent, underestimate the level of exposure. While no specific data exist for phthalates and alkylphenols, pharmacokinetic studies [73] support this possibility. On the other hand, some authors [74–76] indicate that for pregnant women there may be higher values of some phthalates, particularly DEHP, linked to hospitalization and the possible use of drips or catheters. Furthermore, it is documented in the literature how renal clearance is modified during pregnancy [77], and therefore, creatinuria levels generally decrease. It must be said however that in the postpartum days, the levels tend to return to the prepartum ones; therefore, in our case, this specific aspect should not have a significant impact.

There is also the possibility that women during pregnancy have adopted healthier lifestyles which may affect levels of phthalates, even if the questionnaire asked for elements related to possible sources of exposure and many of these do not fall into a bad lifestyle such as makeup or storing fatty foods in plastic containers.

Finally, there are numerous reprotoxic chemicals, and there are further substances that could act on the endocrine system producing adverse effects on reproduction. For

this reason, there is an inevitable limitation of the obtained results, even considering the main confounding factors, and there could be exposures due to particular lifestyle habits that could affect the results.

5. Conclusions

The results of this epidemiological study suggest that exposure to DEP and DEHP, among other substances, may play a role in the RPL and idiopathic infertility. Other substances like BPA or DnBP have shown, with different magnitudes, a higher level in women with specific infertility factors, and this calls for further research to better clarify their role.

These findings highlight the urgent need for more epidemiological investigations focused on idiopathic infertility, because of a lack of information in this field and the possible cumulative effects of chemical mixtures on female reproductive health. In fact, the real exposure is hardly to one substance, but usually to a mixture of substances, and these must be taken into consideration for probable cumulative adverse health effects.

Regarding DEP, there is still no sufficient evidence in the scientific literature to support the hypothesis of its role in infertility problems. However, it should be taken in mind that "it is the dose that makes a substance a poison", and since the MEP concentrations found in our samples are very high, more accurate studies should be carried out to exclude or confirm the possible risk due to DEP exposure in women.

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