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# High plasma concentrations of di-(2-ethylhexyl)-phthalate in women with endometriosis

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**BACKGROUND: Emerging evidence suggests a potential role for ubiquitous environmental contaminants in the physiopathology of endometriosis. Di-(2-ethylhexyl)-phthalate (DEHP), the most commonly used plasticizer in flexible polyvinylchloride (PVC) formulations, is a widespread environmental contaminant with potentially adverse effects on fertility in animal models. In the present study, we tested the hypothesis that DEHP and/or and its main metabolite, mono-ethylhexyl phthalate (MEHP), play a role in the pathogenesis of endometriosis. METHODS: Specimens of blood and peritoneal fluid were collected in a group of women with endometriosis ($n$ = 55), and in age-matched control women ($n$ = 24). Concentrations of DEHP and MEHP were measured in plasma and peritoneal fluid by using high performance liquid chromatography (HPLC). Differences between groups were tested using the Fisher's exact test, Wilcoxon-test, and Kruskal–Wallis analysis of variance. RESULTS: Endometriotic women showed significantly higher plasma DEHP concentrations than controls (median 0.57 μg/ml, interquartile range: 0.06–1.23; values range: 0–3.24 versus median 0.18 μg/ml, interquartile range: 0–0.44; values range: 0–1.03; $P$ = 0.0047) and 92.6% of them had detectable DEHP and /or MEHP in the peritoneal fluid. No significant differences in either the DEHP/MEHP plasma concentrations ($P \geqslant 0.31$) or DEHP/MEHP peritoneal fluid concentrations ($P \geqslant 0.66$) were observed in the endometriotic patients as a function of the disease stage at the time of diagnosis. CONCLUSIONS: The present findings showed for the first time an association between DEHP plasma concentrations and endometriosis, suggesting a possible role for phthalate esters in the pathogenesis.**

*Key words:* Di-(2-ethylhexyl)-phthalate/endometriosis/environmental hazards/mono-ethylhexyl phthalate/plasticizers

## Introduction

The function of the normal human endometrium has been shown to be based on cell–cell interactions regulated by cytokines and growth factors under the direction of steroid hormones. Endometriosis, a common cause of female infertility of unknown aetiology, occurs almost exclusively in menstruating women of reproductive age and may result from disruptions of this well-balanced cellular equilibrium (Olive and Schwartz, 1993; Osteen and Sierra-Rivera, 1997). Emerging evidence suggests a possible role for ubiquitous environmental contaminants in the physiopathology of endometriosis. In particular, polyhalogenated aromatic hydrocarbons (PHAH), a class of widespread environmental contaminants including dioxins, have been postulated to be linked to endometriosis (Pauwels *et al*. 2001; Rier and Foster, 2002), and unbalanced expression of sex hormones or growth factors as a consequence of immune response can be recognized as the result of dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin) exposure (Mayani *et al*., 1997; Osteen and Sierra-Rivera 1997). Di-(2-ethylhexyl)-phthalate (DEHP) is the most commonly used plasticizer in flexible polyvinylchloride (PVC) formulations. However, DEHP does not bind with the plastic and leaches with time and use from vinyl products, thus becoming an ubiquitous environmental contaminant (Mayer *et al*., 1972; Giam *et al*., 1978; Griffiths *et al*., 1985; Sharman *et al*., 1994; Bauer and Hermann, 1997) with potential adverse effects both on fertility and reproduction in animal models (Agarwall *et al*., 1985, 1986, 1989; Parmar *et al*., 1986; Lamb *et al*., 1987; Dostal *et al*., 1988; Berman and Laskey, 1993; Laskey and Berman, 1993; Davis *et al*., 1994; Arcadi *et al*.,

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1998; Li *et al*., 1998; US Food and Drug Administration, 2001; Dalgaard *et al*., 2001; Hoyer, 2001; Sharpe, 2001; Kim *et al*., 2002; Park *et al*., 2002).

To date no information exists with regard to reproductive toxicity in humans. Although it is well known that DEHP is the sole phthalate determining reproductive toxicity by similar mechanisms in males and females (Hoyer, 2001), a major concern exists on the potential adverse effect of DEHP exposure on the human male reproductive tract development (US Food and Drug Administration, 2001). Moreover, there is an age-dependency difference in the DEHP toxicity between males and females, with DEHP toxicity occurring at an older age in females than in males. In fact, while evidence for deteriorating male reproduction consists of a hormonal disturbance of the fetal testis development (Sharpe, 2001), in females the potential DEHP target has been shown to be the ovary and its action mainly consists of an alteration of the natural ovulation times, resulting in hypo-estrogenic anovulatory cycles and polycystic ovaries (Davis *et al*., 1994). In the present study, we tested the hypothesis that DEHP and/or its main metabolite mono-ethylhexyl phthalate (MEHP) play a role in the pathogenesis of human endometriosis.

## Materials and methods

### *Subjects*

The study involved a group of fertile women submitted to diagnostic laparoscopy for the evidence of ovarian cysts or to investigate chronic pelvic pain and dysmenorrhoea. Informed consent was obtained from all subjects before inclusion, and the study protocol was approved by the local committees.

Endometriotic patients ($n$ = 35) [age 36.8 ± 6.7 years (median ± SD); age range 22–45 years] at the time of the diagnosis were enrolled. According to the revised American Fertility Society classification of endometriosis (American Fertility Society, 1985), patients were classified in stage 1 ($n$ = 8), stage 2 ($n$ = 9), stage 3 ($n$ = 12), or stage 4 ($n$ = 6). Diagnosis was confirmed by histological examination of the endometriotic lesions. Exclusion criteria were medical treatment for endometriosis or ovarian cyst before the surgery, any surgical procedure in the previous 12 months.

Twenty-four healthy subjects [age 37.8 ± 5.1 years (median ± SD); age range 18–48 years] without known infertility or reproductive diseases served in the study as age-matched controls. Both groups were subdivided according to the follicular and luteal phase of the menstrual cycle.

### *Fluid sampling*

Blood samples were obtained from a peripheral vein the day before the surgical procedure or immediately before anaesthesia for laparoscopy. Peritoneal fluid was obtained by culdocentesis during laparoscopy. All fluid samples were centrifuged at 3000 *g* for 10 min, and aliquots of the supernatants were stored at –20°C until phthalate measurements.

### *Phthalate measurements*

The concentrations of DEHP and its main metabolite were determined by High Performance Liquid Chromatography (HPLC). DEHP was purchased from Sigma-Aldrich (Milan, Italy) and MEHP was synthesized, from phtalic anhydride and 2-ethyl-1-hexanol, using a previously published method (Giam *et al*., 1978). HPLC analysis was carried out using a Waters system (Waters, Milford, MA, USA) composed of the following: a Model 515 pump, a Model 996 photodiode array detector equipped with a Millenium32 software. A Model 7125 sample injector (Rheodyne, Cotati, CA, USA) equipped with a 20 µl loop was used. The analysis was performed on an analytical 4.6×250 mm internal diameter reversed-phase Spherisorb S5 ODS2 (5 µm particle size) column (Waters) protected by a 4.6×20 mm internal diameter (40 µm particle size) disposable Pelliguard pre-column (Supelco, Bellefonte, PA, USA). Separations were performed at room temperature. The mobile phase consisted of a mixture of acetonitrile and 0.1% $H_3PO_4$ (pH 3.0) (90:10, v/v). The mixture was prepared daily, sonicated before use and delivered at a flow rate of 1.0 ml/min. Column eluate was monitored at 230 nm. Stock solutions containing DEHP or MEHP (2 mg/ml) were prepared by dissolving a weighed amount of substance in acetonitrile. Standard solutions were prepared by dilution of the above stock solutions with mobile phase and by varying the concentration in the range 0.05–5 µg/ml. The calibration curves for HPLC analysis were obtained by plotting peak areas versus concentration. The equations, obtained through regression analysis, of data for the above standard solutions (six data from average of a minimum number of five determinations), showed correlation coefficients of 0.9993 (DEHP) and 0.9994 (MEHP) respectively. A total of 200 µl of plasma was added with 400 µl of NaOH 1 mol/l, 100 µl of 50% $H_3PO_4$ and 600 µl of acetonitrile. After each addition, the sample was shaken by vortex for 30 s. After centrifugation for 10 min at 1500 *g*, the supernatant was separated and the residue was again extracted with 600 µl of acetonitrile. After repeated centrifugation, the supernatants collected were evaporated, reconstituted with 400 µl of mobile phase and injected into the chromatograph. The extraction procedure has been tested with standard solutions of DEHP or MEHP in human reference plasma and showed, in the concentration range 0.05–4 µg/ml, a recovery of 100 and 80% for DEHP and MEHP respectively. The elution peaks of MEHP and DEHP were lacking in interferences deriving from other plasma components and were characterized by retention times of 3.4 and 12.4 min respectively. Phthalate measurements were performed by chemical analysts who were unaware of the patients' clinical data.

### *Data analysis*

Data were expressed as mean ± SD for continuous normally distributed data and medians with inter-quartile range (25th and 75th percentiles) for non-normal distributions. Fit to normal distribution of cord blood DEHP and MEHP was tested using the $\chi^2$ two-tailed test. Pairwise differences between groups were assessed using either Fisher's exact test (categorical variables) or Wilcoxon's test (continuous variables). Differences between DEHP/MEHP concentrations and endometriosis stage were assessed using the Kruskal–Wallis one-way analysis of variance. Correlation analysis between the DEHP and MEHP concentrations and continuous biological variables was performed with the use of Spearman's correlation coefficient ($r_s$). The MedCalc version 7.0 statistical software package (Mariakerke, Belgium) was used. A two-sided $P$-value of <0.05 was considered to indicate statistical significance.

## Results

Detectable DEHP and/or MEHP were found in 55/59 (93.2%) of the total serum samples (DEHP and MEHP was detectable in endometriotic samples 32/35 = 91.4% versus controls 23/24 = 95.8%), and in 25/27 (92.6%) of the peritoneal fluids of women with endometriosis.

Distributions of DEHP and MEHP plasma ($\chi^2$ = 65.3 and 253.27 respectively; $P < 0.0001$) and peritoneal ($\chi^2$ = 70.7 and

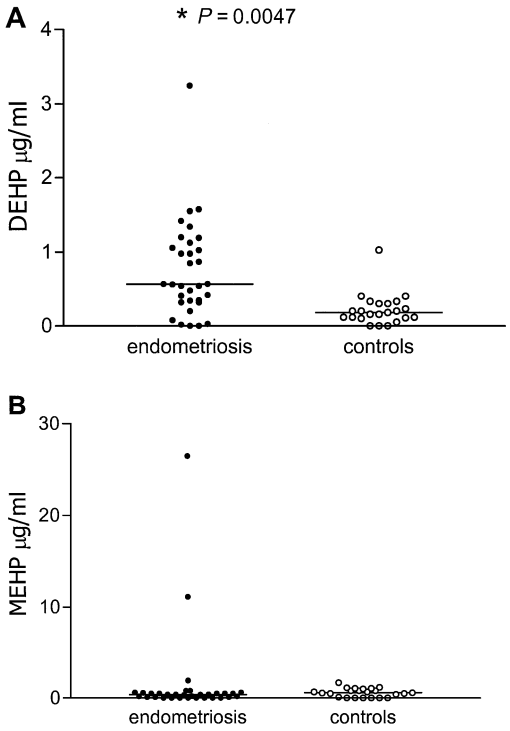


**Figure 1.** Plasma concentrations of DEHP (**A**) and MEHP (**B**) in patients with endometriosis versus healthy controls.

87.2 respectively; $P < 0.0001$) concentrations were significantly different from gaussian. Endometriotic patients showed significantly higher plasma DEHP concentrations than controls (median 0.57 µg/ml, interquartile range: 0.06–1.23; values range: 0–3.24 versus median 0.18 µg/ml, interquartile range: 0–0.44; values range: 0–1.03; $P = 0.0047$) (Figure 1). Plasma MEHP levels were comparable between groups (median 0.38 µg/ml, interquartile range: 0.1–0.97; values range: 0–26.47 versus median 0.58 µg/ml, interquartile range: 0.34–0.71; values range: 0–1.69; $P = 0.12$).

DEHP and MEHP peritoneal fluid concentrations were median 0.46 µg/ml (interquartile range: 0.21–0.69; values range: 0–11.7) and median 0.37 µg/ml (interquartile range: 0–0.58; values range: 0–11.82). No differences in either DEHP /MEHP plasma concentrations (H = 3.52, $P = 0.31$; and H = 1.95, $P = 0.56$ respectively) or DEHP/MEHP peritoneal fluid concentrations (H = 1.59, $P = 0.66$; and H = 0.94, $P = 0.81$ respectively) were observed as a function of either disease stage or cycle phase (plasma DEHP, $P = 0.72$; plasma MEHP, $P = 0.26$; peritoneal fluid DEHP, $P = 0.42$; peritoneal fluid MEHP, $P = 0.21$).

No statistically significant relations were observed between DEHP and MEHP concentrations ($r_s \leqslant 0.16$; $P \geqslant 0.31$) or between DEHP/MEHP concentrations and age ($r_s \leqslant 0.39$, $P \geqslant 0.12$).

## Discussion

Little is known about the aetiology and pathogenesis of endometriosis, considered to be a complex multifactorial disease (Olive and Schwartz, 1993), although emerging evidence suggests a potential pathogenetic role for environmental exposure to PHAH. Environmental toxins may directly (hormone disruptor) or indirectly (immune toxin) affect the response of the endometrium to steroids, resulting in endometriosis (Osteen and Sierra-Rivera, 1997).

The present findings indicate a previously unrecognized association between DEHP plasma levels and endometriosis. Phthalate esters are a class of water-insoluble organic chemicals that have been used as plasticizers for polyvinylchloride (PVC) formulations since about 1930. They are widely used in many applications, including medical devices, toys, food wraps, shoe soles and interior building surfaces and therefore are ubiquitous environmental contaminants (Latini and Avery, 1999; Latini 2000; Tickner *et al*., 2001; US Food and Drug Administration, 2001; Health Canada Expert Advisory Panel on DEHP, 2002). DEHP and MEHP have been shown to have detrimental effects on fertility and reproduction in experimental animal models. In particular, DEHP has been suspected of possessing endocrine disruptor properties, by antagonizing the effects of sex hormones or altering the actions of endogenous steroid hormones, in laboratory animals (Agarwal *et al*., 1985; 1986, 1989; Parmar *et al*., 1986; Lamb *et al*., 1987; Dostal *et al*., 1988; Berman and Laskey, 1993; Laskey and Berman, 1993; Davis *et al*., 1994; Arcadi *et al*., 1998; Li *et al*., 1998; US Food and Drug Administration, 2001; Dalgaard *et al*., 2001; Hoyer, 2001; Sharpe, 2001; Kim *et al*., 2002; Park *et al*., 2002). However, there is concern that these compounds may be causing adverse effects on human reproductive health (Latini, 2000; Tickner *et al*., 2001; US Food and Drug Administration, 2001; Health Canada Expert Advisory Panel on DEHP, 2002). Accordingly, the Health Canada Expert Advisory Panel on DEHP in medical devices has reported that research into alternatives to DEHP-containing products should be urgently encouraged (Health Canada Expert Advisory Panel on DEHP, 2002).

The reasons for the observed differences in the relative DEHP/MEHP concentrations in the examined body fluids between endometriotic and control women remain to be elucidated. In particular, the volume distributions of DEHP/MEHP in the various human body compartments during health and diseases are unknown to date. The association here observed is biologically plausible since (i) a relationship between endometriosis and environmental contaminants other than DEHP has been already evidenced (Mayani *et al*., 1997; Osteen and Sierra-Rivera, 1997; Rier and Foster, 2002; Pauwels *et al*., 2001); and (ii) although major concern is to date related to potential adverse effects on male infertility (Agarwal *et al*., 1986; Parmar *et al*., 1986; Lamb *et al*., 1987; Dostal *et al*., 1988; Arcadi *et al*., 1998; Li *et al*., 1998; Dalgaard *et al*., 2001; Hoyer, 2001; Sharpe, 2001; Park *et al*., 2002; US Food and Drug Administration, 2001; Health Canada Expert Advisory Panel on DEHP, 2002), DEHP has proven detrimental effects on fertility and reproduction in female animal models (Agarwal *et al*., 1985; 1989; Lamb *et al*., 1987; Berman and Laskey 1993; Laskey and Berman 1993; Davis *et al*., 1994; Kim *et al*., 2002). In particular, DEHP has been shown to hinder the development of reproductive organs and an anti-estrogenic action of DEHP has been proposed as its cause

(Lamb *et al*., 1987). The anti-estrogenic action of phthalates seems to occur through a receptor-mediated signalling pathway able to suppress estradiol production in the ovary, leading to anovulation (Lovekamp-Swan and Davis, 2003).

Further studies are needed in order to elucidate the mechanisms underlying the observed statistical association.

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