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Influence of polycyclic aromatic hydrocarbon exposure on IVF: now is the time to focus on women

Antoine Netter[1,2,*], Elena Siri[1,2], Virginie Tassitro[2], Noémie Resseguier[3], Nicolas Beauval[4], Irène Sari-Minodier[2,5], Blandine Courbiere[1,2], Jeanne Perrin[2,6]

ABSTRACT

Research question: Is polycyclic aromatic hydrocarbon (PAH) exposure associated with the reproductive outcomes of IVF treatment?

Design: A prospective, small-scale monocentric cohort study of couples who underwent IVF treatment between January 2018 and June 2019. Both members of each couple answered a questionnaire on PAH exposure and provided urine samples to measure urinary 1-hydroxypyrene (1-OHP) the day before oocyte retrieval and semen collection for fertilization. To assess the specific PAH exposure of gamete cells, immunostaining was conducted on both spermatozoa and granulosa cells obtained during IVF with an anti-benzo(a)pyrene diol epoxide (BPDE) monoclonal antibody that recognizes BDPE-DNA adducts. To assess DNA damage, a comet assay on spermatozoa was conducted. The PAH exposure was compared between couples who had positive HCG and couples who had negative HCG on day 14 after embryo transfer.

Results: Eighteen couples were included. The mean 1-OHP level in women whose HCG tests were positive (n = 6) was significantly lower than that in women with negative HCG tests (0.098 [0.042–0.170] versus 0.177 [0.067–0.812] µg/g creatinine; P = 0.048). The presence of BPDE-DNA adducts in granulosa cells of women with a negative (29.7 [16.2–57.5] arbitrary units) or positive HCG test (20.3 [9.3–23.3] arbitrary units) were not significantly different (P = 0.092). The urinary 1-OHP levels of men and BPDE-DNA adducts in spermatozoa showed no differences between groups.

Conclusions: This exploratory research should encourage further studies to determine the effect of women's exposure to PAHs on reproductive outcomes of IVF treatment.

[1] Department of Gynecology, Obstetrics and Reproductive Medicine, AP-HM La Conception, Pôle femmes parents enfants, 147 bd Baille, Marseille 13005, France
[2] Aix Marseille Univ, Avignon Université, CNRS, IRD, IMBE, Marseille, France
[3] Laboratoire de santé publique (EA 3279) (public health laboratory), faculté de Médecine de la Timone, 27, boulevard Jean Moulin, Marseille cedex 5 13385, France
[4] CHU Lille, Unité Fonctionnelle de Toxicologie, Lille F-59000, France
[5] Service hospitalo-universitaire de médecine et santé au travail, Faculté des Sciences Médicales et Paramédicales, AP-HM La Timone, Aix Marseille Univ 27, boulevard Jean Moulin, Marseille Cedex 5 13385, France
[6] Laboratoire de Biologie de la Reproduction - CECOS, Pole Femmes-Parents-Enfants, AP-HM La Conception, Marseille, France

KEYWORDS

Biomarkers
Comet assay
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INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous and recalcitrant pollutants produced by incomplete combustion of organic substances (*Alegbeleye et al., 2017*). Polluted air, occupational exposure, cigarette smoke and charbroiled or contaminated food are exposing millions of humans to PAH (*Zhang et al., 2008*; *Liu et al., 2012*). Sixteen PAH are designated priority pollutants for monitoring by the European and US environmental protection agencies for their carcinogenic and mutagenic properties (*European Parliament, Council on Environmental Quality Standards in the Field of Water Policy, 2008*; *US Environmental Protection Agency, 2014*).

Data on the effect of PAH exposure on human reproduction are scarce. The results of several studies have suggested that the exposure of men to PAH is associated with DNA damage in spermatozoa without interfering with semen parameters. In men, exposure to PAH might therefore be related to infertility and might not be diagnosed by a common semen analysis (*Zenzes et al., 1999a*; *Gaspari et al., 2003*; *Han et al., 2011*; *Perrin et al., 2011*; *Jeng et al., 2013*; *Yang et al., 2017*). In-vivo studies in mice have identified several mechanisms that explain how exposure to PAH could damage spermatozoa DNA (*Revel et al., 2001*; *Inyang et al., 2003*; *Ramesh et al., 2004*; *2008*). The transformation of PAH into epoxide metabolites that bind covalently to DNA and induces a stable primary lesion (termed a DNA adduct) is the most unanimously accepted mechanism (*Zenzes et al., 1999a*; *Revel et al., 2001*; *Baird and Ralston, 2004*; *Perrin et al., 2011*). In addition, PAH epoxide metabolites are formed *via* a microsomal cytochrome P450-dependent monooxygenase system that is known to increase the production of reactive oxygen species and potentially damage DNA (*Park et al., 1996*; *Lopes et al., 1998*).

Data on the effect of PAH exposure on female fertility are even more scarce. In-vivo animal studies have suggested that the PAH affect the ovarian follicles and oocyte and cumulus cell DNA through similar mechanisms than for spermatozoa (*Bengtsson et al., 1983*; *Neal et al., 2007*; *Ramesh et al., 2010*; *Sobinoff et al., 2012*; *Einaudi et al., 2014*). Because of obvious difficulties obtaining human ovarian cells, to date, only one study has investigated the effect of PAH on female fertility. In 1998, *Zenzes et al. (1998)* revealed the formation of BPDE-DNA adducts in the granulosa cells of 32 women undergoing IVF treatment who were exposed to cigarette smoke (*Zenzes et al., 1998*). The same team found more BPDE-DNA adducts in the embryos of smoking couples than in those of non-smoking couples, suggesting that DNA damage caused by PAH exposure could be transmitted to the embryos. The reproductive outcomes of IVF, however, were not reported in that study (*Zenzes et al., 1999a*).

Over recent decades, increasing research has been conducted to identify the best practices to increase the chances of pregnancy with IVF treatment (*Kushnir et al., 2017*). The influence of lifestyle and environmental exposure on the outcomes of IVF have increasingly been researched (*Hornstein, 2016*; *Vizcaíno et al., 2016*; *Choe et al., 2018*). Despite the presumption of impairment of human gametes owing to PAH exposure, however, to the best of our knowledge, nothing is known on its influence on the success of assisted reproductive technology (ART) treatments.

The aim of this small-scale prospective, monocentric, cohort study was to determine whether PAH exposure among women and men was associated with the outcomes of IVF treatment.

MATERIALS AND METHODS

Study design

A small-scale prospective, monocentric, cohort study was conducted in our ART unit at La Conception University Hospital in Marseille, France. Data were prospectively and anonymously collected. The study was approved (20 June 2018) by the Ouest II Committee for the Protection of Research Subjects (RCB: 2018-A00364-51). Women and men from each couple provided written informed consent. The trial is registered with ClincalTrials.gov (reference: NCT03914859).

Study population

Couples who underwent IVF treatments in our ART unit were enrolled between February 2018 and June 2019. Couples were eligible for inclusion if the woman was aged between 18 and 36 years, had a regular menstrual cycle of between 27 and 33 days, had not had more than one other IVF attempt and if the man's age was between 18 and 45 years. Exclusion criteria were IVF with gamete donation, American Fertility Society revised stage III or IV (moderate or severe) endometriosis (*Revised American Society for Reproductive Medicine classification of endometriosis, 1997*), decreased ovarian reserve (AMH <1 ng/ml), medical history likely to alter either ovarian reserve, fertilization rate or implantation, e.g. history of cancer with chemotherapy or radiotherapy for either member of the couple, uterine or ovarian surgery, and positive human immunodeficiency virus, hepatitis C virus or hepatitis B virus serologies for any member of the couple.

Ovarian stimulation protocols and luteal phase support

The ovarian stimulation protocols and luteal phase support used in our ART unit have been described previously (*Netter et al., 2019*). Briefly, either gonadotrophin releasing hormone agonist or gonadotrophin releasing hormone antagonist protocols were used. Either recombinant FSH, recombinant FSH and recombinant LH or human menopausal gonadotrophin was used for ovarian stimulation. A single injection of 250 µg of choriogonadotropin alpha (Ovitrelle, Merck Serono Europe Limited, UK) was used for final follicular maturation. Oral dydrogesterone (Duphaston®, Mylan Medical, France) was used for luteal phase support.

Polycyclic aromatic hydrocarbon exposure questionnaires and expired carbon monoxide measurement

On the morning of oocyte retrieval, both members of the couple answered a questionnaire assessing tobacco consumption and dietary, environmental and occupational exposures to PAH. The questionnaire used in this study was strongly inspired by the questionnaire created by *Gunier et al. (2006)* for a study on PAH exposure. Questions on occupational exposure to PAH were added by using parts of another questionnaire created and published by our team in 2009 (*De Fleurian et al., 2009*). The first section of the questionnaire briefly focused on exposure the day before oocyte retrieval, and the second section extensively focused on exposure in day to day life (**Supplementary Table 1**). Participants were considered occupationally exposed

to PAH if they answered 'yes' to one or more options in question B.1.3. ('In the workplace, are you exposed to…?). The participants were considered to be exposed to PAH by their diet if they declared that they ate, smoked or grilled meat or fish several times a week or every day (question B.2.1.). The participants were considered environmentally exposed to PAH if they answered 'yes' to any of the options in the following questions: B.1.4. ('Is your workplace located in a large city?'), B.1.5. ('Is your workplace located near the following locations…?'), B.2.4.1. ('Is your place of residence located in a large city?'), B.2.4.2. ('Is your residence located near the following locations…?'), and B.2.4.3. ('Do you use the following heating methods…?').

To counter the risk of underestimating tobacco consumption, both members of the couple underwent an expired carbon monoxide measurement on the morning of oocyte retrieval using a piCO Smokerlyzer carbon monoxide breath test monitor according to the manufacturer's instructions (Bedfont Scientific Ltd, Harrietsham, Maidstone, UK). The results are reported in parts per million (ppm).

Urine samples and 1-hydroxypyrene measurement

Both members of the couple were asked to provide urine samples at the end of the day preceding oocyte retrieval. When possible, oocyte retrievals were not carried out on Mondays so that the urine samples were always produced in the evening of a working day to better assess occupational exposure. Five milliliters of each urine sample were immediately collected to measure the creatinine concentration; then, the samples were stored at –80°C and kept away from light until analysis. The 1-OHP measurements were conducted at the toxicology unit of Lille University Hospital, France. Briefly, after β-glucuronidase hydrolysis, urine samples successively underwent several solvent extraction, drying and reconstitution steps adapted from a published method (*Li et al., 2005*). They were then analysed by ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (Xevo TQ-XS, Waters, Guyancourt, France). The lower limit of quantification for 1-OHP was 0.025 µg/l, and 1-OHP concentrations were adjusted to urinary creatinine. Samples with creatinine concentrations over 3.0 or less than 0.3 g/l, considered over-concentrated and over-diluted, respectively, were excluded (*Leroyer et al., 2010*).

Sperm and follicular cell collection and storage

Semen samples were collected by masturbation for IVF on the day of oocyte retrieval after a period of abstinence of 2–6 days. Semen analysis was carried out after 30 min of liquefaction at 37°C, and smears were stained with Schorr staining in accordance with the current World Health Organization guidelines (*WHO, 1999*). Sperm morphology defects were assessed according to the method described by *David et al. (1975)* and modified by Jouannet *et al* (*Jouannet et al., 1988*).

Follicles were monitored by ultrasound during ovarian stimulation. On the day of oocyte retrieval, all follicles were aspirated 36 h after HCG administration using transvaginal guidance and local or general anaesthesia. Oocytes were then retrieved from the follicular fluid under microscope examination.

Informed consent was obtained for inclusion of the remaining sperm cells and the granulosa cells remaining in follicular fluid in the Germetheque Biobank, France, after freezing in liquid nitrogen (*Di Santo et al., 2012*).

Comet assay and immunostaining for anti-benzo(a)pyrene diol epoxide DNA adducts

After thawing, spermatozoa were selected by a swim-up technique already described by our team (*Perrin et al., 2011*). Alkaline comet assay was carried out according to the procedure described by *Singh et al. (1988)* and adapted by *Baumgartner (2009)* for human spermatozoa, which has already been described in other works by our team (*Préaubert et al., 2018*). The percentage of DNA in the tail and the olive tail moment of 100 randomly selected sperm cells from each slide (in triplicate per patient) was reported.

After thawing, the immunostaining and BPDE-DNA adduct quantification procedures were the same for spermatozoa and granulosa cells and have been described elsewhere (*Perrin et al., 2011*). The method used an anti-benzo[a]pyrene diol epoxide (BPDE) monoclonal antibody that recognizes BDPE-DNA adducts. For the men, 100 spermatozoa were randomly selected for each slide in duplicate for the analysis, and 50 granulosa cells were chosen for women. The fluorescence was expressed as arbitrary units (arb. Units) of average grey value average.

End-points

The HCG serum level was measured 14 days after embryo transfer and considered positive if HCG was greater than 100 IU/ml. The primary end-points were differences in markers of exposure to PAH (urinary 1-OHP, answers to questionnaires, expired carbon monoxide and BDPE-DNA adducts immunostaining in germ cells for each member of the couple) between the couples for whom the implantation occurred (positive HCG on day 14 after embryo transfer) and those for whom implantation did not occur (negative HCG at day 14 after embryo transfer). The secondary end-points were the correlation coefficients between the markers of exposure to PAH and embryo quality, semen analysis and DNA damage to spermatozoa assessed by the comet assay. Embryo quality was assessed by the Plachot and Mandelbaum classification considering the number, symmetry and fragmentation of blastomeres depending on the day of development (*Plachot and Mandelbaum, 1990*). Top-quality embryos were those presenting four regular blastomeres on day 2 and 8, regular blastomeres on day 3 with less than 10% fragments, and no multinucleated blastomeres (*Plachot and Mandelbaum, 1990*).

Statistical analysis

Data are presented as numbers and percentages for categorical variables and as means and SD for quantitative variables or as medians and minimum–maximum based on their distribution.

The sample size was estimated under the following hypotheses: a minimum effect size of 1.5; a positive or negative HCG ratio of 33% based on previously published results from our ART unit (*Netter et al., 2019*); a bilateral alpha risk of 0.05; and a power of 0.80. With these criteria, a sample size of 18 couples was required (*Machin et al., 2009*). Considering that some couples could be excluded from the analysis, the sample size was increased, and 22 couples were recruited. First, two groups were determined based on the results of HCG on day 14 after embryo transfer. Clinical and biological characteristics were compared between these two groups. Quantitative data were compared using Student's t-tests when applicable and using Mann–Whitney

U-tests otherwise. The normality of the distribution of quantitative data was checked by Levene's test. Categorical data were compared using chi-squared test when applicable and Fisher's test otherwise.

Second, correlations were examined between the markers of exposure to PAH and the quality of the highest-grade embryo transferred, semen analysis, and DNA damage to spermatozoa assessed by the comet assay. Results are reported as Spearman rank correlation coefficients.

All tests were two-sided, and results were considered to be significant when $P < 0.05$. IBM SPSS Statistics 20.0 (IBM Inc., New York, USA) was used for all statistical analyses.

RESULTS

Study population and reproductive outcomes

During the study period, 22 couples undergoing IVF treatment at our ART unit were included in the study. Four couples did not have embryo transfer on day 2 after oocyte retrieval (three for ovarian hyperstimulation syndrome and one for failure to obtain embryos of sufficient quality) and were excluded from the final analysis. Among the 18 remaining couples, six (33%) had positive HCG, and 12 (67%) had negative HCG on day 14 after embryo transfer. Characteristics of women and embryological outcomes are presented in TABLE 1. Characteristics of men and semen parameters are presented in TABLE 2.

Association between women's polycyclic aromatic hydrocarbon exposure and reproductive outcomes and embryo quality

A comparison of women's PAH exposure between positive and negative HCG groups is presented in TABLE 3. Women who had negative HCG on day 14 after embryo transfer had significantly higher median urinary 1-OHP (0.177 [0.067–0.812] µg/g creatinine) compared with women who had positive HCG (0.098 [0.042–0.170] µg/g creatinine; $P = 0.048$). The presence of BPDE-DNA adducts in granulosa cells of women with a negative HCG test (29.7 [16.2–57.5] arbitrary units) compared with women with a positive HCG test (20.3 [9.3–23.3] arbitrary units) were not significantly different ($P = 0.092$). Occupational, environmental and dietary exposures to PAH were similar in both groups.

The correlations between women's PAH exposure markers and the quality of the highest-grade embryo transferred are presented in FIGURE 1. Women's urinary 1-OHP was significantly positively correlated to the degree of fragmentation of the highest-grade embryo transferred (Spearman's rho r = 0.489, $P = 0.040$). The correlation between the BPDE-DNA adducts in granulosa cells and the degree of fragmentation of the highest-grade embryo transferred was not significant (r = 0.290, $P = 0.416$). No correlation was found between either the women's urinary 1-OHP or the BDPE-DNA adduct and the embryo score of the highest-grade embryo transferred or the chances for the highest-grade embryo transferred to be a top-quality embryo. Smoking status was strongly negatively correlated with the chances for the highest-grade embryo transferred to be a top-quality embryo (r = –0.535, $P = 0.022$). Expired carbon monoxide did not show any correlation with any of the criteria of embryo quality.

Association between men's polycyclic aromatic hydrocarbons exposure and reproductive outcome and embryo quality

Comparisons of men's characteristics between positive and negative HCG groups are presented in TABLE 4. Occupational exposure to PAH was significantly more frequent in the negative HCG group (50%) than in the positive HCG group (0%; $P = 0.034$). Nevertheless, neither the men's urinary 1-OHP nor the BDPE-DNA adducts in spermatozoa were different between groups ($P = 0.562$ and $P = 0.385$, respectively). Sperm DNA damage assessed by the comet assay was not significantly different between groups. Dietary and environmental exposure to PAH were similar between groups.

The correlations among men's PAH exposure markers and semen quality and sperm DNA damage assessed by the comet assay are presented in FIGURE 2. Men's urinary 1-OHP levels were not correlated with either semen parameters or sperm DNA damage. The correlation between sperm BPDE-DNA adducts and sperm DNA fragmentation parameters was not significant (r = 0.335; $P = 0.174$ for the percentage tail DNA and r = 0.337; $P = 0.123$ for the olive tail moment).

The correlations among men's PAH exposure markers and embryo quality parameters are presented in FIGURE 3. Men's

TABLE 1 WOMEN'S CHARACTERISTICS AND EMBRYOLOGICAL OUTCOMES ACCORDING TO HCG TEST RESULT ON DAY 14 AFTER EMBRYO TRANSFER

Characteristics	n	Positive HCG	Negative HCG
Patients, n	18	6	12
Maternal age, years	18	32 ± 4	33 ± 3
Maternal BMI, kg/m^2	18	23.4 ± 3.5	24.5 ± 4.3
Basal AMH, IU		2.2 (1.5–5.14)	2.0 (0.8–6.7)
Primary diagnosis for infertility	18		
Unexplained, n (%)		2 (33)	5 (42)
Tubal, n (%)		3 (50)	4 (33)
Endometriosis, n (%)		0 (0)	0 (0)
PCO/PCOS, n (%)		0 (0)	1 (8)
Male, n (%)		1 (17)	2 (17)
mature oocytes retrieved, n	18	11 (7–25)	8 (3–18)
Fertilization rate, %	18	84 (67–100)	67 (25–100)
Number of embryos transferred, n	18	1.3 ± .5	1.8 ± .4
At least one top quality embryo transferred, n (%)	18	5 (83)	4 (33)
Frozen embryos, n	18	2.5 (0 – 9)	0 (0 – 4)
Maternal smoking, n (%)	18	0 (0%)	4 (33%)
Expired carbon monoxide, ppm	18	2 (2–10)	2.5 (1–8)

Descriptive data are presented as mean ± SD for quantitative data, or as median (minimum–maximum), according to their distribution. Categorical data are presented as numbers (%).

AMH, anti-Müllerian hormone; PCO, polycystic ovaries; PCOS, polycystic ovary syndrome.

TABLE 2 COMPARISON OF MEN'S CHARACTERISTICS AND SEMEN PARAMETERS ACCORDING TO HCG TESTING ON DAY 14 AFTER EMBRYO TRANSFER

Characteristics	n	Positive HCG	Negative HCG
Patients, n	18	6	12
Paternal age, years	18	33 ± 4	36 ± 5
Paternal BMI, kg/m^2	18	24.7 ± 3.3	26.1 ± 1.9
Semen volume, ml	18	3.3 (.9 – 4.5)	3.4 (2.2 – 5.8)
Sperm concentration, G/ml	18	35.6 (5.9 – 99.5)	75.0 (6.1 – 284)
Sperm motility, %	18	37.5 (30 – 55)	30 (20 – 55)
Sperm vitality, %	17	68 (64 – 82)	77 (54 – 88)
Sperm normal morphology, %	17	19 (2 – 26)	29 (8 – 44)
Paternal smoking, n (%)	18	3 (50)	3 (25)
Carbon monoxide breath test (ppm)	18	5 (2–11)	3 (1–11)

Descriptive data are presented as mean ± SD for quantitative data, or as median (minimum–maximum), according to their distribution. Categorical data are presented as numbers (%).

TABLE 3 COMPARISON OF WOMEN'S POLYCYCLIC AROMATIC HYDROCARBON EXPOSURE ACCORDING TO HCG TESTING ON DAY 14 AFTER EMBRYO TRANSFER

Characteristics	n	Positive HCG	Negative HCG	*P*-value
Patients, n	18	6	12	
Occupational PAH exposure, n (%)	18	0 (0)	0 (0)	1.0
Dietary PAH exposure, n (%)	18	3 (50)	5 (42)	0.737
Environmental PAH exposure, n (%)	18	4 (67)	5 (42)	0.317
Urinary 1-OHP (µg/g creatinine)	18	0.098 (0.042–0.170)	0.177 (0.067–0.812)	0.048[a]
Granulosa cell BPDE-DNA adducts (arbitrary unit)	10	20.3 (9.3 – 23.3)	29.7 (16.2 – 57.5)	0.092

[a] $P < 0.05$.Descriptive data are presented as median (minimum–maximum). Categorical data are presented as numbers (%).BPDE, benzo(a)pyrene diol epoxide; PAH, polycyclic aromatic hydrocarbon; 1-OHP = 1-hydroxypyrene.

urinary 1-OHP did not correlate with any of the parameters of embryo quality. The correlation between spermatozoa BPDE-DNA adducts and embryo fragmentation was not significant ($r = 0.386$; $P = 0.114$). No correlation was found between sperm BPDE-DNA adducts and the embryo score of the highest-grade embryo transferred or with the chances for this embryo to be a top-quality embryo. Neither tobacco consumption nor expired carbon monoxide were correlated with any of the parameters used to assess embryo quality.

DISCUSSION

To the best of our knowledge, this small-scale study is the first study to investigate the association between PAH exposure and the reproductive outcomes of IVF treatment. The urinary 1-OHP levels in women were positively correlated with fragmentation of the highest- grade embryo transferred and were significantly higher in women with a negative HCG test result 14 days after embryo transfer compared with women with a positive result. This finding suggests an association between PAH exposure in women and reduced quality of the highest-grade embryo transferred conceivably linked to the absence of implantation. The women's tobacco consumption had a negative effect on the quality of the highest-grade embryo transferred, but this effect was not mediated through the fragmentation of

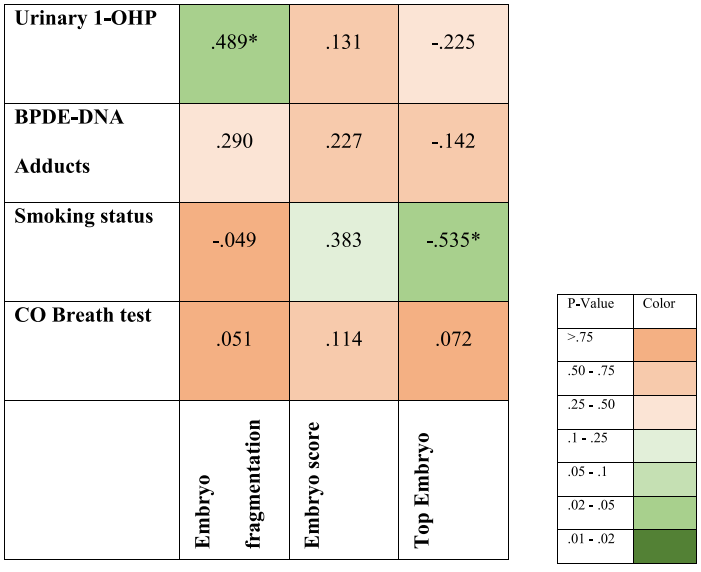


FIGURE 1 Correlations between the women's exposure markers and the quality criteria of the highest-grade embryo transferred. The values in the figure show the Spearman's correlation coefficient estimates (rho) with their *P*-values indicated by colour. Embryo fragmentation is the fragmentation of the highest-grade embryo transferred assessed with a discrete variable with four possible ranges (<10%, 10–30%, 30–50% and >50%). Embryo score is the score of the highest-grade embryo transferred assessed by the Plachot and Mendelbaum classification from 1 (best quality) to 4 (worst quality). Top embryo is the chances for the highest-grade embryo transferred to be a top-quality embryo (binary variable). [a]$P < 0.05$. BPDE, benzo(a)pyrene diol epoxide; 1-OHP = 1-hydroxypyrene.

TABLE 4 COMPARISON OF MEN'S POLYCYCLIC AROMATIC HYDROCARBON EXPOSURE AND SPERM DNA DAMAGE ACCORDING TO HCG TESTING ON DAY 14 AFTER EMBRYO TRANSFER

Characteristics	n	Positive HCG	Negative HCG	*P*-value
Patients, n	18	6	12	
Occupational PAH exposure, n (%)	18	0 (0)	6 (50)	0.034[a]
Dietary PAH exposure, n (%)	18	4 (67)	6 (50)	0.502
Environmental PAH exposure, n (%)	18	2 (33)	6 (50)	0.502
Men's urinary 1-OHP (μg/g creatinine)	18	0.147 (0.053–0.259)	0.134 (0.058–0.284)	0.562
Spermatozoa BPDE-DNA adducts (arbitrary unit)	18	15.1 (10.1 – 32.0)	13.4 (6.0 – 28.5)	0.385
Comet assay	18			
Sperm tail DNA (%)		38.1 (22.6–50.1)	28.4 (21.5–49.8)	0.204
Olive tail moment		5.4 (3.6–10.6)	4.2 (3.1–9.8)	0.535

[a] $P < 0.05$.Descriptive data are presented as median (minimum–maximum). Categorical data are presented as numbers (%).BPDE, benzo(a)pyrene diol epoxide; PAH, polycyclic aromatic hydrocarbon; 1-OHP = 1-hydroxypyrene.

embryos. Notably, although the effect of smoking on fertility has been investigated in a range of studies, these studies mostly focused on ovarian reserve, semen quality and uterine receptiveness. Therefore, the effect of tobacco consumption on the quality of embryos in IVF treatment has been minimally and inconsistently investigated (*Freour et al., 2008*). In contrast, our results suggest that PAH exposure in men has a reduced effect on the implantation rate than women's exposure.

In 1999, *Zenzes et al. (1999b)* revealed that BPDE-DNA adducts could be transmitted through gametes to human preimplantation embryos (*Zenzes et al., 1999b*). As the investigators found similar staining intensity in embryos from couples in which both members smoked than in embryos from couples in which only the father smoked, they concluded that the contribution of modified DNA to the embryo was mainly through the spermatozoa. As the investigators acknowledged, however, this conclusion had to be confirmed by running the same analyses on embryos from couples in which only the mother smoked to presumably show that the staining would be lower. Such couples were not recruited in their study, and no other similar study has been conducted subsequently. Therefore, since the publication of this report, it has been presumed that BPDE-DNA adducts are transmitted mostly through the spermatozoa and that women's exposure to PAH is less likely to alter the DNA of embryos, even though the scientific evidence was insufficient. Although our study did not investigate the immunostaining of embryos, our results suggest that an association exists between PAH exposure in women and the fragmentation and, more importantly, implantation of embryos. Therefore, we hypothesize that the assumption of a lesser importance of women in the transmission of BPDE-DNA adducts to the embryos could be erroneous. Further studies involving immunostaining of embryos should be conducted to confirm this hypothesis.

To date, research on the effect of PAH on human reproduction has almost exclusively focused on men's fertility and were mostly conducted in conditions of high exposure (either in emerging countries, heavily industrialized cities affected with significant air pollution or with occupationally exposed subjects) (*Hsu et al., 2006*; *Xia et al., 2009*; *Han et al., 2011*; *Jeng et al., 2013*; *Jurewicz et al., 2013*; *Yang et al., 2017*). This study was original in that it considered the exposure of both members of the

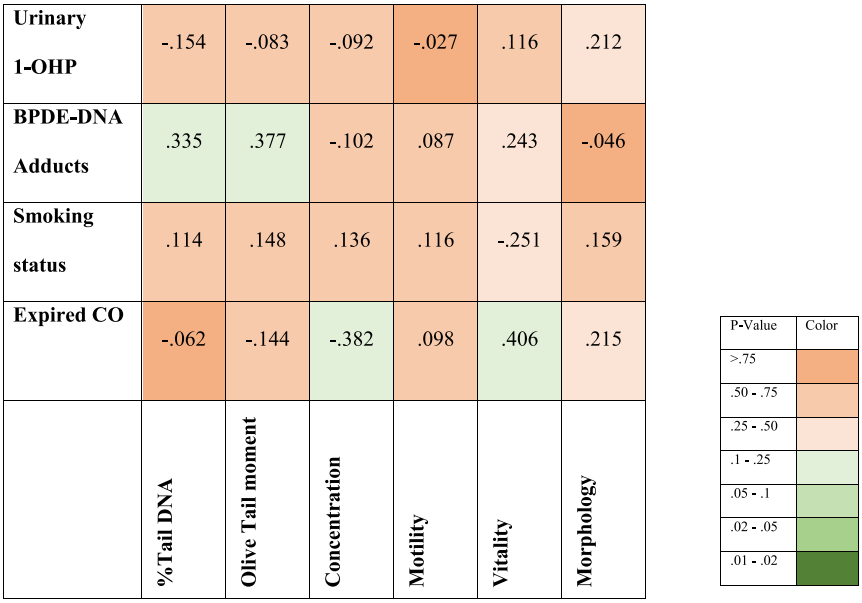


FIGURE 2 Correlation of men's exposure markers with semen parameters and sperm DNA damage (assessed by the comet assay). The values in the figure show the Spearman's correlation coefficient estimates (rho) with their *P*-values indicated by colour. BPDE, benzo(a)pyrene diol epoxide; 1-OHP = 1-hydroxypyrene.

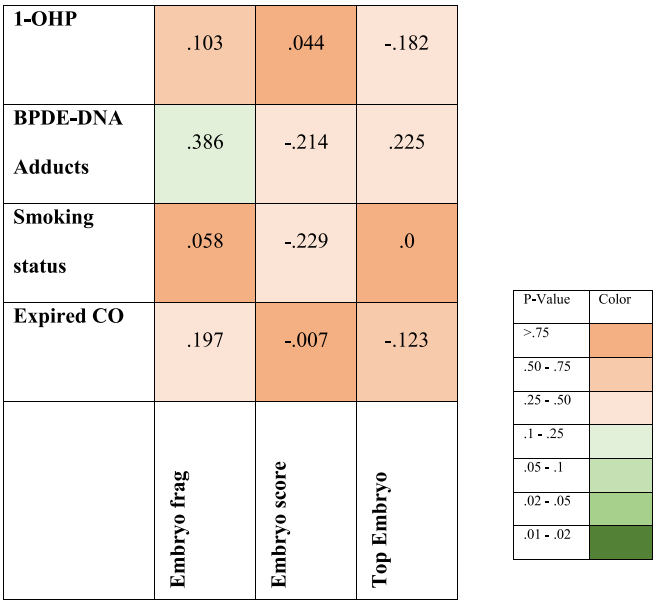


FIGURE 3 Correlations between men's exposure markers and the quality criteria of the highest-grade embryo transferred. The values in the figure show the Spearman's correlation coefficient estimates (rho) with their *P*-values indicated by colour. Embryo fragmentation is the fragmentation of the highest-grade embryo transferred assessed with a discrete variable with four possible ranges (<10%, 10–30%, 30–50% and >50%). Embryo score is the score of the highest-grade embryo transferred assessed by the Plachot and Mendelbaum classification from 1 (best quality) to 4 (worst quality). Top embryo is the chances for the highest-grade embryo transferred to be a top-quality embryo (binary variable).

couple and that it was conducted in a region mildly affected by PAH pollution. Therefore, our results are more likely to generalize to other fertility clinics in high-income countries. The underlying goal of this study, assuming that its results are confirmed by subsequent larger studies, was to ultimately determine a specific threshold for 1-OHP or other PAH metabolites above which the implantation of the embryo is unlikely to occur, thereby justifying postponement of the IVF stimulation cycle until the exposure factor is identified and eliminated. These thresholds could be different from those that are currently used for monitoring professional exposure as they were not measured to predict implantation.

The most obvious limitation of the present study was the small sample size. The low number of patients exposed (only three women and no men had urinary 1-OHP levels above 0.3 µg/g creatinine) did not allow us to create useful groups of exposure, and we were unable to propose a suitable and specific cut-off. We carefully selected our population only to include couples that were not exposed to other factors that would alter the chances of implantation. Our original intention was to obtain a cohort of couples for whom the absence of pregnancy could not be easily explained by any other obvious cause, e.g. advanced age, endometriosis and decreased ovarian reserve. To this end, we mostly retained couples for whom the primary diagnosis was either unexplained or tubal infertility. Although this rigorous method adds reliability to our results, it also dramatically prolonged the study period to almost 18 months to include the required number of couples. The lesson to be learned from this small-scale study is that further research on the matter should be conducted in collaboration with other ART units to include enough patients in a reasonable amount of time. The interpretation of our results is also limited by a possible confounding bias as the sample size and the data available were insufficient to conduct a reliable logistic regression analysis. In particular, it is impossible to rule out the influence of other pollutants as co-exposures are common. Indeed, the PAH exposure could be associated with the exposure to other substances that could negatively influence the reproductive outcomes on their own or in synergy with the PAH. To overcome this bias in further studies, other biomarkers should also be measured.

Another limitation to this study was that we only measured one PAH urinary metabolite. Although 1-OHP is the most commonly used urinary biomarker for estimating exposure to PAH, most of the studies on the matter rely on various sets of PAH metabolites to obtain a more accurate representation of different parent compounds (*Castano-Vinyals et al., 2004*; *Han et al., 2011*). Further studies on the effect of PAH on IVF should be conducted with a larger set of urinary metabolites and with repeated measurements over time to assess the exposure more accurately.

In conclusion, although this exploratory research should be interpreted with caution, it should encourage further studies to determine the effect of PAH exposure of couples and of the exposure of women on reproductive outcomes of IVF treatment, in particular.

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