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ORIGINAL ARTICLE

Polycyclic aromatic hydrocarbons exposure and early miscarriage in women undergoing *in vitro* fertilization-embryo transfer

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ABSTRACT

Various factors have been reported to be associated with early miscarriages, including microbial infection, chemical toxicity, maternal disorders and genetic abnormalities. In the present study, a prospective cohort study was conducted to investigate whether urinary concentrations of hydroxylated polycyclic aromatic hydrocarbons (OH-PAHs) were associated with early pregnancy loss in women undergoing *in vitro* fertilization-embryo transfer (IVF-ET). Risk of spontaneous pregnancy loss in patients exposed to PAHs was analysed using 40 patients who had experienced early pregnancy loss compared to 40 who had normal live births. Single spot morning urine samples were collected from each patient 30 days after embryo transfer when clinical pregnancy was confirmed and ten urinary OH-PAHs were measured. After adjustment for age and BMI using a Log Binomial Model, only 2 + 3-PHE was found to be associated positively with early miscarriage. Using Receiver Operating Characteristic (ROC) curve analysis, the Area Under the Curve (AUC) was 0.78 ($p<0.001$), suggesting that 2 + 3-PHE might provide a potential biomarker to predict the miscarriage risk in patients exposed to high level of PAHs.

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Introduction

It has been estimated that approximately one-third of all pregnancies end in early miscarriage in the first trimester (Wilcox et al., 1988), and numerous studies indicate that a variety of factors, including microbial infection, chemical toxicity, maternal disorders and genetic abnormalities, are involved (Arck et al., 2008; Buss et al., 2006). Exposure to environmental endocrine disruption agents may lead to first trimester pregnancy loss due to the sensitivity of embryonic tissue to such compounds. Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous toxic pollutants in the environment formed through incomplete combustion of organic materials, such as coal, wood, gas, oil and tobacco products (Bai & Zhang, 2016; Bansal & Kim, 2015). The main routes of human exposure are ingestion of food containing PAHs, inhalation of polluted air and cigarette smoke, while occupational exposure is mainly via dermal absorption (Bostrom et al., 2002; Hansen, Mathiesen, Pedersen, & Knudsen, 2008; Zhang et al., 2014). After PAHs enter the body, they undergo two metabolic phases. In phase I metabolism, PAHs are oxidized by cytochrome P450 enzymes to form reactive intermediates which may be hydrolyzed to hydroxylated metabolites. Under phase II metabolism, the hydroxylated PAHs (OH-PAHs) are conjugated with glucuronic acid or sulphate and excreted through the urine or faeces (Jacob & Seidel, 2002). OH-PAHs have short half-lives, estimated to be from 2.5h for 2-hydroxynaphthalene (2-NAP) to 23.5h for 1-hydroxypyrene (1-PYR) (Bai & Zhang, 2016; Fan et al., 2012).

PAH exposure is considered to pose harmful health effects in man; many PAHs are known mutagens and carcinogens with an increasing number of studies reporting skin, lung and bladder cancers (Armstrong, Hutchinson, Unwin, & Fletcher, 2004; Bostrom et al., 2002; Roelofzen et al., 2012). Recent studies also suggest that PAHs have adverse effects on reproductive health as endocrine disrupters (Bai & Zhang, 2016; Peterson et al., 2015). For example, men who smoke, or are exposed to PAHs through their occupation, have reduced sperm counts and motility (Jeng, Pan, & Chao, 2013; Jeng, Pan, Chao, & Lin, 2015; Madeen &

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Williams, 2016). Maternal exposure to PAHs may lead to intrauterine growth restriction (Choi, Rauh, Garfinkel, Tu, & Perera, 2008) and preterm birth (Singh et al., 2007). A dose-response relationship between PAHs in placenta and the risk of neural tube defects has been observed (Fan et al., 2012; Langlois et al., 2012) and intrauterine exposure of PAHs may dysregulate foetal ovarian developmental signalling (Fowler et al., 2014). However, it is not clear whether high urinary PAH concentrations affect early reproductive outcomes in women adversely, especially those undergoing *in vitro* fertilization-embryo transfer (IVF-ET).

In this study, we conducted a prospective cohort study to examine the influence of maternal exposure to PAHs on spontaneous early pregnancy loss in patients who conceived following IVF-ET. Ten urinary OH-PAHs were tested.

Materials and methods

Study participants and data collection

This was a prospective cohort study. 369 infertile patients undergoing IVF/ICSI cycle were recruited in the Centre for Reproductive Medicine of Nanfang Hospital, Southern Medical University from October 2015 to May 2016. Of these, 207 patients conceived after fresh or frozen embryo transfer. The risk of spontaneous pregnancy loss in patients exposed to PAHs was analysed using 40 patients who experienced early pregnancy loss compared to 40 normal live births randomly selected from the cohort. The study was approved by the Institutional Review Board for Nanfang Hospital (study number NFEC-2015-106), and all the participants gave written informed consent. Clinical information was obtained from electronic medical records.

Upon recruitment, a brief questionnaire was given to each patient to collect baseline data including demographics, occupation, medical history, lifestyle and residential factors. All the participants were screened to exclude smokers and passive smokers (a person exposed to smoking at work or at home at least 1 day per week for over 15 min). Women with chronic diseases were also excluded. These included hypertension, heart diseases, diabetes, thyroid dysfunction and any disease that might be associated with spontaneous early pregnancy loss.

Treatment protocol and clinical IVF measures

Patients were treated with a standard long GnRH-agonist protocol. After pituitary down-regulation, ovarian stimulation was conducted until at least two follicles had reached a diameter of $\geq$18 mm, followed by human chorionic gonadotropin (HCG) administration to induce final follicular maturation. Oocyte retrieval was performed 36h later by trans-vaginal ultrasound-guided follicle aspiration. Sperm samples were obtained by masturbation after 3–5 days abstinence from subfertile men with normozoospermia. Motile spermatozoa were prepared by gradient centrifugation and incubated with oocytes for fertilization 4 hours after oocyte retrieval. Subsequent embryos were cultured individually from the time of fertilization until assessment on day 3.

A maximum of three embryos were transferred on day 3 after retrieval and luteal phase support was given by daily injections of progesterone started from the day of oocyte retrieval continuing until the day of pregnancy test. A clinical pregnancy was defined as an intrauterine gestational sac with heartbeat 30 days after embryo transfer. Early miscarriage was defined as spontaneous pregnancy loss prior to 13 weeks gestation.

Blood samples and hormone assays

Serum hormone levels were measured to assess the possible endocrine disruptive effects of PAHs. Blood samples were taken on day 2–5 of the patients' spontaneous menstrual cycle before IVF treatment for baseline measurements of follicle stimulating hormone (FSH), luteinizing hormone (LH), oestradiol (E2), progesterone (P4). Serum peak E2 level was measured on the day of HCG administration. Serum hormonal levels were measured by electrochemiluminescence (ECL) immunoassay (Roche, USA).

Urine sample collection and urinary PAHs metabolites concentrations

Single spot morning urine samples were collected from each patient 30 days after embryo transfer when clinical pregnancy was confirmed. 15 mL of fasting urine samples were collected in a sterile clean polypropylene bottle. Each urine sample was divided into aliquots and creatinine measured immediately. The remaining aliquots were stored at –20 °C away from light.

The urinary concentrations of total (free and conjugated) OH-PAHs were measured using solid-phase extraction (SPE) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The limit of detection (LOD) was 0.006–0.034 μg/L and the limit of quantification (LOQ) was 0.018–0.10 μg/L. Creatinine concentrations were measured by an automated analyzer. Creatinine concentrations in samples $>$3.0 or $<$0.3 g/L were excluded. OH-PAH concentrations were

adjusted by creatinine (Cr) concentrations (μmol/L). OH-PAH concentrations below the LOD were assigned a value equal to the LOD divided by the square root of 2 before Cr adjustment. Method accuracy and precision were evaluated by repeated analysis of matrix spiked at three levels of 1.0 μg/L, 5.0 μg/L and 10.0 μg/L for ten OH-PAHs. Recoveries were 85–131% with relative standard deviations (RSDs) of 3–23%.

Statistical methods

Demographic and baseline reproductive characteristics of the female patients were presented using mean ± SD or percentages, and OH-PAHs concentrations of different groups were presented using medians (Q1–Q3). Demographic and baseline reproductive characteristics between the two groups of patients were compared using Student's *t*-test for continuous variables. A Log Binomial Model, reported to be more robust than Logistic Regression Model for common outcomes with incidence higher than 10% (Greenland, 2004), was used to evaluate the association between urinary OH-PAHs concentrations and pregnancy outcomes. Since age and BMI (Turner et al., 2010) are risk factors associated with early miscarriages, they were included as covariates in the final model. Receiver-operator characteristic (ROC) analyses were performed with urinary OH-PAHs concentrations plotted against early pregnancy loss. The area under the ROC curve (AUC) was used to assess the predictive power. The aims of conducting ROC analysis were to examine the model performance as well as to establish the ideal cut-off points for the covariates of interest to predict pregnancy outcome.

All tests were two-tailed and the level of statistical significance was set at 0.05. Student's *T*-test and ROC analysis were performed using SPSS statistical software version 12.0.1 and Log Binomial Model was performed using SAS statistical software version 9.4.

Results

Demographics

Patients' demographic characteristics and the early outcomes of *in-vitro* fertilization (IVF) are summarized in Table 1. The average age, BMI and baseline FSH were slightly higher while the total antral follicle count, peak E2 level on the day of HCG administration and the number of oocyte retrieved were lower in the case participants than the controls but without statistical significance ($p > 0.05$). Furthermore, the oocyte maturation rate, fertilization rate, normal fertilization rate and highest quality embryo formation were slightly lower in the case participants but without any statistical significance ($p > 0.05$, Data not shown). Overall, no significant difference could be observed between the two groups of patients either in basic clinical features or early outcomes of IVF-ET ($p > 0.05$).

Table 1. Patients' demographic characteristics were compared in case and reference group analyzed by *t-test*.

	Early miscarriage ($n = 40$) (mean ± SD)	Live birth ($n = 40$) (mean ± SD)	*p*
Mean age (years)	30.12 ± 4.14	29.87 ± 6.15	0.09
Body mass index (BMI) (kg/m^2)	21.35 ± 2.80	21.27 ± 2.27	0.90
Total antral follicle count	14.72 ± 7.02	15.78 ± 10.06	0.64
Baseline FSH (mIU/mL)	7.52 ± 3.79	6.82 ± 1.61	0.38
Baseline LH (mIU/mL)	5.59 ± 4.51	6.18 ± 5.47	0.86
Baseline LH/FSH (mIU/mL)	0.86 ± 0.60	0.96 ± 0.93	0.60
Baseline PRL (ng/mL)	16.48 ± 7.46	18.21 ± 12.53	0.53
Baseline T (ng/mL)	0.25 ± 0.14	0.26 ± 0.16	0.80
Baseline E2 (pg/mL)	42.56 ± 18.82	38.85 ± 14.73	0.41
Total dose of gonadotrophins (IU)	2484.03 ± 1219.59	2276.42 ± 1027.41	0.48
E2 on HCG (pg/mL)	2752.51 ± 1467.37	3646.89 ± 2615.56	0.12
Number of oocytes retrieved	11.67 ± 6.51	13.73 ± 9.37	0.31
Number of MII oocytes	10.22 ± 5.30	12.42 ± 9.08	0.28
Number of fertilized eggs	9.03 ± 4.98	10.38 ± 9.17	0.50
Number of 2PN eggs	6.75 ± 3.98	8.19 ± 6.88	0.34
Number of top quality embryos	3.03 ± 2.06	3.96 ± 4.06	0.29

Exposure levels of urinary PAHs

The distribution of creatinine adjusted urinary OH-PAH levels (μmol/mol Cr) in 80 patients are shown in Table 2. All the compounds were detectable in both groups of patients. 1-, 2-NAP, and 2 + 3-FLU contributed 85.87% of the total concentration of all OH-PAHs (∑OH-PAHs), while 4-PHE and 1-PYR only accounted for 0.94% and 2.54% respectively. ∑OH-PAHs covered a range of 0.47–22.69 μmol/mol Cr with a median level of 1.95 μmol/mol Cr. The sum of PHE metabolites (1-, 2-, 3-, 4-, 9-PHE) was in the range 0.38–13.28 μmol/mol Cr with a median level of 1.01 μmol/mol Cr.

Urinary OH-PAHs concentrations and early pregnancy loss

The urinary concentrations of 2 + 3-FLU, 2 + 3-PHE, 1 + 9-PHE, 1-PYR, as well as ∑OH-PAHs were significantly higher in patients who experienced early pregnancy loss ($p < 0.05$, Table 3). However, after adjustment for age and BMI using Log Binomial Model, a significant association could only be observed between 2 + 3-PHE and early pregnancy loss ($PR = 6.82$, $p = 0.04$, Table 4). An ROC analysis was therefore performed with urinary 2 + 3-PHE concentrations plotted against early miscarriage (Figure 1). The area under the curve (AUC) was 0.78 ($p < 0.001$). The optimal cut-off

Table 2. Distribution of urinary creatinine adjusted urinary OH-PAHs levels (μmol/mol Cr).

Compound	Geometric mean	Arithmetic mean (SD)	25th centile	50th centile	75th centile
2-NAP	1.80	2.51 (2.34)	0.94	1.58	3.43
1-NAP	0.75	1.68 (3.14)	0.38	0.71	1.57
2 + 3-FLU	0.44	0.72 (0.92)	0.21	0.47	0.78
2 + 3-PHE	0.21	0.35 (0.48)	0.12	0.23	0.41
1 + 9-PHE	0.17	0.26 (0.32)	0.10	0.17	0.28
4-PHE	0.03	0.05 (0.15)	0.01	0.03	0.05
1-PYR	0.10	0.15 (0.19)	0.06	0.09	0.16
∑OH-PAHs	2.12	3.19 (3.86)	1.07	1.95	3.39

Table 3. Urinary OH-PAHs levels of cases and reference group (μmol/mol Cr) by *t*-test.

	Live birth ($n = 40$)	Early miscarriage ($n = 40$)	
	(Median (Q1–Q3))	(Median (Q1–Q3))	*p*
2-NAP	1.89 (1.01–3.30)	1.20 (0.82–3.71)	0.89
1-NAP	0.67 (0.32–1.50)	0.92 (0.44–1.85)	0.31
2 + 3-FLU	0.35 (0.20–0.67)	0.61 (0.31–1.16)	0.05
2 + 3-PHE	0.15 (0.06–0.25)	0.36 (0.22–0.66)	0.01
1 + 9-PHE	0.12 (0.09–0.23)	0.24 (0.12–0.50)	0.03
4-PHE	0.02 (0.01–0.03)	0.04 (0.03–0.07)	0.14
1-PYR	0.07 (0.05–0.11)	0.15 (0.08–0.21)	0.02
∑OH-PAHs	1.46 (1.01–2.54)	2.18 (1.61–5.47)	0.04

Table 4. OH-PAHs exposure and early pregnancy loss after adjusted for age and BMI using Log Binomial Model.

Outcomes	PR	95% CI	*p*
Age	0.18	0.03–0.33	0.01
BMI	−0.14	−0.43–0.15	0.35
2-NAP	0.01	−0.32–0.35	0.94
1-NAP	0.03	−0.22–0.29	0.80
2 + 3-FLU	0.09	−0.12–0.32	0.42
2 + 3-PHE	6.82	0.35–13.29	0.04
1 + 9-PHE	−2.03	−10.68–6.62	0.65
4-PHE	20.75	−29.34–70.84	0.42
1-PYR	19.64	−5.70–44.99	0.13

value of 2 + 3-PHE concentration for predicting early pregnancy loss was 0.24 μmol/mol Cr with maximum values of sensitivity and specificity of 73% and 72%, respectively.

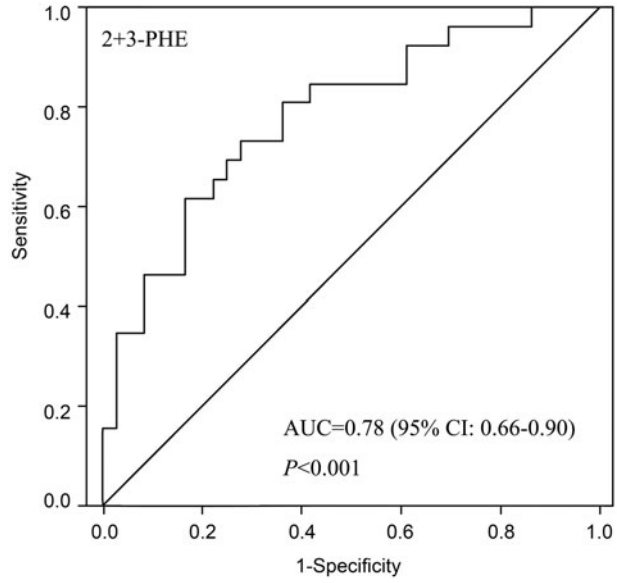

Figure 1. ROC curve for 2 + 3-PHE for the prediction of early pregnancy loss. AUC for 2 + 3-PHE is 0.78 ($p < 0.001$). AUC: area under the curve; CI: confidence interval.

Discussion

Ten PAH metabolites were detectable in all the participants, but the urinary concentrations of some compounds were relatively lower than the levels reported in previous studies (Fan et al., 2012). This was probably due the subjects being children from polluted and non-polluted area in Guangzhou in Fan et al.'s study, while the subjects were general population in the study of Guo et al. (2013). The subjects in the present study were quite different since they were subfertile and seeking assisted reproduction. They understood the harm of pollutants and made efforts to avoid living in polluted areas (places with heavy traffic or with factories nearby). Moreover, it has been reported that children have a mean PAH level 30% higher than adults who live in the same environment (Huang, Caudill, Grainger, Needham, & Patterson, 2006). It was therefore reasonable that the urinary concentration was lower in the present study in which 1-, 2-NAP, and 2 + 3-FLU contributed 85.87% of the total urinary OH-PAH concentration, which was similar to the ambient air PAH profiles in Guangzhou (Armstrong et al., 2004), indicating that inhalation of polluted air might be the major route of exposure.

As early as 1990, PAH-DNA adducts could be detected in placenta and fetal tissues obtained from human spontaneous abortions (Hatch, Warburton, & Santella, 1990), and various studies have indicated that PAH exposure might be associated with early miscarriage (Detmar & Jurisicova, 2010; Detmar et al., 2006; Wu, Hou, Ritz, & Chen, 2010). However, most previous studies focused on the effects of cigarette smoking with only benzo pyrene (BaP), benz and anthracene being analysed (Armstrong et al., 2004). In the present study, smokers, passive smokers and patients with

chronic diseases were excluded, and the concentration of low molecular weight OH-PAHs (two to four ring PAHs) were analyzed, which were mostly traffic-related pollutants. The urinary concentrations of 2 + 3-FLU, 2 + 3-PHE, 1 + 9-PHE, 1-PYR, and ∑OH-PAHs were significantly higher in patients suffering from early pregnancy loss, indicating the possible adverse health impacts of high levels of environmental PAHs. After adjustment for age and BMI using Log Binomial Model, a positive association could be observed between 2 + 3-PHE and early pregnancy loss. Since 2 + 3-PHE correlated well with other OH-PAHs, an ROC analysis was therefore conducted. The AUC was 0.78 with a maximum value of sensitivity and specificity of 73% and 72%, respectively at the optimal cut-off value of 2 + 3-PHE (0.24 μmol/mol Cr), which indicated that the urinary concentration of this compound might be used to predict the risk of early miscarriages in patients exposed to high level of PAHs.

Various PAHs have been reported to have endocrine-disrupting activities through oestrogen signalling pathways (Zhang, Dong, Wang, Tao, & Kiyama, 2016). Chronic exposure of so-called 'produced water' i.e. water trapped in underground formations that is brought to the surface during oil or gas exploration and production, which is a complex mixture of PAHs and alkylphenols produced in offshore oil and gas drilling processes, may lead to a significant decrease of oocyte number in fish such as polar cod (Geraudie, Nahrgang, Forget-Leray, Minier, & Camus, 2014), and postnatal exposure to PAHs [BaP, benz, anthracene and benzo, and fluoranthene (Armstrong et al., 2004)] revealed ovarian disruptive effects in rats (Kummer, Mašková, Zralý, & Faldyna, 2013). Although in this study, no significant differences could be observed between the two groups of patients in basic reproductive characteristics or early outcomes of IVF-ET, a large sample-sized cohort study needs to be conducted to delineate the relationship between PAH exposure and early miscarriage in women undergoing IVF-ET.

The potential limitations of our study were the small sample size and the single spot sampling method. Considering the short half-lives of OH-PAHs in humans, urinary OH-PAHs represent human exposure to PAHs in the short term such that single spot sampling may lead to misclassification of PAH exposure. Further research is needed discover the pathogenesis of PAH exposure in early pregnancy loss.

In conclusion, there were adverse associations between urinary OH-PAH concentrations and early pregnancy outcomes in patients undergoing IVF-ET. Patients who experienced early miscarriage had significantly higher urinary OH-PAH concentration compared to the normal live birth group. Preliminary analysis showed that 2 + 3-PHE might be used as a potential biomarker to predict the miscarriage risk in patients exposed to high level of PAHs. Further research on the reproductive disrupting effects of PAHs is still needed.

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