

The risk of missed abortion associated with the levels of tobacco, heavy metals and phthalate in hair of pregnant woman

A case control study in Chinese women

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Abstract

To assess the association between exposure to the tobacco, heavy metals and phthalate on early pregnancy and missed abortion.

42 women with missed abortion and 57 matched controls (women with normal pregnancies) were recruited between March and May 2012, from the Department of Gynecology and Obstetrics, First Affiliated Hospital of Guangxi Medical University and the People Hospital of Guangxi Zhuang Autonomous Region. The questionnaire survey was carried on to learn about the basic conditions, as well as smoking history of all participants. The levels of tobacco, heavy metal, and phthalate exposure were compared between the 2 groups by measuring nicotine, cocaine, cadmium (Cd), manganese (Mn), plumbum (Pb) and dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), di-2-ethyl hexyl phthalate (DEHP) in the hair samples.

Our results showed that significant differences in age ($P = .042$), premarital examination ($P = .041$), passive smoking ($P = .021$), and heavy metal exposure ($P = .022$) were found in the case group compared to the control. In addition, the concentration of nicotine ($P = .037$), cotinine ($P = .018$), Cd ($P = .01$), Pb ($P = .038$) and DEHP ($P = .001$) in the hair were significantly higher in the case group. Furthermore, logistic analysis revealed that age [Odds Ratio (OR) 1.172, 95% confidence interval (CI) 1.036–1.327], Cd (OR 8.931, 95% CI 2.003–39.811), Cotinine (OR 4.376, 95% CI 1.159–16.531), DEHP (OR 1.863, 95% CI 1.103–3.146) were important factors contributing to the missed abortion ($P < .05$).

It was demonstrated that high gestational age, passive smoking, heavy metals, and the phthalate exposure were the risk factors for missed abortion, while the premarital health examination was a protective factor. Avoiding these harmful substances before getting pregnant and during the early stages of pregnancy, might help prevent missed abortions.

Abbreviations: BBP = butyl benzyl phthalate, Cd = cadmium, CI = confidence interval, DBP = dibutyl phthalate, DEHP = di-2-ethyl hexyl phthalate, DEP = diethyl phthalate, DiBP = di-iso-butyl phthalate, DMP = dimethyl phthalate, DnBP = di-n-butyl phthalate, EEC = The European Economic Community, hCG = human chorionic gonadotropin, LOD = limit of detection, MA = missed abortion, Mn = manganese, OECD = The Organization for Economic Co-operation and Development, OR = odds ratio, Pb = plumbum.

Keywords: heavy metals, missed abortion, phthalate exposure, tobacco

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1. Introduction

Missed abortion (MA), also known as silent miscarriage, usually occurs when an embryo or fetus dies, but the body does not recognize the pregnancy loss and continues to release hormones.^[1] MA is very common complication, which affects ~15% of all clinically recognized pregnancies.^[2] The loss of a pregnancy is often distressing for women and their partners, with adverse effects on their social and psychological wellbeing.^[3] In China, the incidence of MA has been rapidly increasing (13.4%) presenting itself as an important public health problem.^[4]

The pathogenesis of MA has not yet been fully understood, and it is commonly believed that the combination of multiple factors may lead toward this outcome. The main known cause of MA involves genetic factors, such as chromosomal abnormalities, that is, the number of chromosomes or their structural abnormalities.^[5] The other risk factors for MA include an abnormal intrauterine environment, endocrine disorders, immune dysfunction, excessive smoking, severe systemic infections, nutritional deficiencies, environmental chemicals, and trauma.^[6–8] Over the years, environmental pollution has become another important risk factor for MA. It has been shown that the ambient air pollutants may influence the progress of the pregnancy and fetus growth, and may lead to poor birth outcome. Furthermore,

exposure to harmful chemical and physical factors have shown strong association with cessation of embryonic development, which may consequently lead to MA. In this study, we investigated the association of tobacco, heavy metals, and phthalates exposure with MA. During the early pregnancy, harmful substances abundantly present in the tobacco smoke, such as nicotine and cotinine, reduce progesterone secretion, and may consequently lead to spontaneous abortion. Phthalates are a plastic plasticizer widely used in plastic industry for medicinal products, food packaging, personal care products, and polyvinyl chloride.^[9,10] As environmental endocrine disruptors, phthalates have shown to have estrogen activity, which can induce the reproductive and developmental toxicity.^[11]

Urine or blood levels are usually used to assess the chemical exposure. However, a lot of chemicals have a short half-life, therefore the level of these chemicals in urine or blood may not reflect the actual whole-body exposure. For this reason, hair samples might provide a better option. Hair is a biological sample that is easy to collect, transport, and storage. Additionally, hair has certain enrichment of metal elements, so it is widely used for trace elements analysis.^[12] Moreover, compared to blood or urine samples, hair has substantially longer detection window (month to year) which may enable retrospective investigation of chronic and past consumption.^[13] This memory effect the hair has due to accumulation of chemicals provides possibility to examine exposure to variety of pesticides (including different chemical categories such as organochlorines, organophosphates, pyrethroids, and other), polybrominated diphenyl ethers, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and phthalates.^[12,14] Consequently, this study uses the hair as biological sample to assess the exposure to tobacco, heavy metals, and phthalates.

In the following paper, we measured the levels of tobacco and heavy metals exposure by examining nicotine and cotinine, and plumbum, cadmium and manganese, respectively, in hair samples. We measured the exposure to heavy metals by detecting plumbum (Pb), cadmium (Cd), manganese(Mn) in hair samples. In addition, we analyzed the exposure to phthalates by testing phthalic acid esters, including dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), and di-2-ethyl hexyl phthalate (DEHP) in the same hair samples.

2. Methods

2.1. Study population

Pregnant women, <20 weeks' gestation, age between 19 and 46 years, were recruited from the Department of Obstetrics and Gynecology of Guangxi Medical University Affiliated Hospital and the People Hospital of Guangxi from March to May 2012. All study subjects received a detailed description of the study protocol and signed the informed consent approved by the institutional review board of Guangxi Medical University. Trans-vaginal ultrasound examination was used to obtain real-time visual images of the *developing embryo* or fetus, empty gestational sac or an embryo/fetus without cardiac activity was used as diagnosis criteria for miss abortion.^[15] The pregnant woman who was diagnosed as miss abortion was recruited as MA group. The pregnant women having ectopic pregnancy, hydatidiform mole, twin pregnancy, gynecological inflammation, and reproductive tract abnormalities were excluded from the study. Finally, 42 patients with MAs and 57 controls

completed the questionnaire and provided hair samples. The case group and control groups were matched by ethnologic and gestation length.

Each subject completed a questionnaire survey which included subject's general information (age, weight, gestational age, ethnologic data, etc.), previous drugs consumption, harmful factors exposure (heavy metals, pesticide, chemical agent, etc.) and partner's general situation (age, smoking, drinking, work environment, etc.). Additionally, the questionnaire had question about frequency and quantity for tobacco, heavy metals, and plastic products exposure during one year before pregnancy and during early stages of pregnancy.

Pregnant women hair samples were cut from occipital clingy scalp using clear stainless steel scissors. Since hair grows at a rate of ~13 mm per month,^[16] assay of a 3.9 cm length of hair close to scalp covered the early gestational period. The hair samples were put into clean bag and tagged.

2.2. Tobacco, heavy metal, and phthalate exposure assessment

The concentration of nicotine, cotinine in the hair samples was analyzed using gas chromatography (6890N, Agilent, America), according to the following method: hair samples were cleaned by detergent (pH=7.0), tap water, and deionized water, and then left to dry out. Samples were then cut into 1 to 2 mm long using a clear stainless steel scissors. Hair samples (~0.48–0.52 g) were then placed in a glass tube, by adding 5.00 mL NaOH (2M) and 20 μ L quinolone internal standard (0.109 mg/mL). The samples were ultrasonicated for 30 minutes and incubated for 4 hours at $40 \pm 1^\circ\text{C}$ for digestion. After incubation, 5.00 mL methanol–methylene chloride mixed solvent (1:3, V: V) was added into the tube. The process to transfer nicotine and cotinine from the digestion liquid to the organic phase was done by vortexing the sample for 2 minutes and then transferring 2.50 mL organic extract solution into a glass centrifuge tube. Around 20 μ L glacial acetic acid was added. The mix solution was dried by nitrogen. The dry sample was added 0.20 mL methanol and blander vortex mixed for 1 minutes, finally centrifuged with 2000 rpt/min for 5 minutes.

Detection of phthalates was done as follows: 0.1 g of hairs samples were transferred in a glass tube containing 1.0 mL 2 mol/L NaOH solution. The tube was then placed in 37°C constant temperature bath box for 2.5 hours to digest adequately. The digested liquid was added in 1 mL methylene chloride extraction solvent. After blending, the mixed liquid was extracted using ultrasonic method for 30 minutes, and then centrifuged. Finally, phthalates were analyzed by gas chromatography.

For hair heavy metal analysis, ~0.5 g hair samples were added to a sample cup containing 5 mL of $\text{HNO}_3\text{--H}_2\text{O}_2$ (3:1, V: V). The mixture was pretreated at 100°C for 30 minutes using multipurpose pretreatment device (XT-9800, Shanghai Xintuo analytic instrument science and technology Ltd.). After cooling, the mixture was then digested for additional 8 minutes on 0.1 MPa using an optical fiber pressure control closed microwave digestion system (MK-III, Shanghai Xinke Microwave Digestion Sample Testing Technology Research Institute). Consequently, the sample was volatilized acid at 160°C in the pretreatment of heating apparatus to nearly dry, then transferred to 25 mL volumetric flask and constant volume with deionized water. The concentration of Mn in the digestion liquid was determined by flame method using atomic absorption spectrophotometer (AA-6800F, Shimadzu, Japan). 5 mL of the digestion liquid was added

0.2 mL of 0.1 g/mL $(\text{NH}_4)_2\text{SO}_4\text{-KH}_2\text{PO}_4$ matrix modifier, then with the deionized water to capacity 10 mL. The concentration of Pb and Cd was determined by graphite furnace method using atomic absorption spectrophotometer (AA6300C, Shimadzu, Japan).

2.3. Statistical analysis

The differences between groups were analyzed with Student's *t*-test for continuous variables and the chi-square test for categorical variables. The odds ratio (OR) associated with cigarette smoking, heavy metals, and phthalates exposure were calculated by comparing the control group with the case group.

All variables identified on the basis of biologic plausibility and preliminary analyses as possible confounders were included in logistic-regression models, which were then simplified with the use of a backward-elimination technique. All final models included the same set of statistically significant covariates and each final model also included questionnaire survey and hair analysis of tobacco exposure, heavy exposure, and phthalates exposure. Logistic regression analysis was used to compare MA group and the control group for all potential factors, including the background information, environmental, and behavioral factors. Odds ratio (OR) and 95% confidence interval (CI) were used to estimate the associations between the underlying factors and the MA. Statistical significance was defined as $P < .05$. All statistical analyses were performed with SPSS 17.0 (SPSS, Chicago, IL).

3. Results

3.1. Participant characteristics

Participant's baseline characteristics and exposure factors data were obtained using the questionnaire survey (Table 1). Briefly, the results showed that the average age of women who suffered from MA (case group) was slightly higher compared to the women with normal pregnancy (control group) ($P < .05$). In addition, controls were more likely to have premarital health check-up compared to the case group ($P < .05$). Furthermore, the exposure to passive smoking and heavy metals during pregnancy was significantly higher in the case group compared to a control group ($P < .05$); while no difference in levels of education, race, active smoking, infection, plasticizer contact during pregnancy, etc. were observed between the 2 groups ($P > .05$).

3.2. Comparison of concentration in the case and control groups

Different levels of nicotine, cotinine, Mn, Ca, Pb, DMP, DEP, DBP, BBP, and DEHP from the participant's hair samples are shown in Table 2. In order to find the liminal value of the risk factors, we used several cutoff values to compare the risk factor levels. The obtained results showed higher levels of nicotine ($P = .037$) and cotinine ($P = .018$) in the case group compared to the control group. These data re-confirmed results obtained elsewhere, nicotine and cotinine are risk factors for MA.^[19] A total of 0.5 $\mu\text{g/g}$ levels of nicotine and the 1 $\mu\text{g/g}$ levels of cotinine found in control group were higher compared to case group. However, exposure to high levels of nicotine ($>0.5 \mu\text{g/g}$) and cotinine ($>1 \mu\text{g/g}$), the proportions of case group (23.81%, 28.57%) were significantly higher compared to the control group (7.02%, 12.28%). Additional, no significant changes in the Mn level were observed between the groups ($P > .05$); while the levels

Table 1

Characteristics of the study subjects.

Characteristics	Case group	Control group	P
Age, years*	28.76 ± 5.165	26.70 ± 4.728	.042
Education (n)			.331
<12 years	11 (26.2%)	9 (15.8%)	
12 years	13 (31.0%)	16 (28.1%)	
>12 years	18 (42.8%)	32 (56.1%)	
Race (n)			.948
Han	22 (53.4%)	28 (49.1%)	
Zhuang	16 (38.1%)	23 (40.4%)	
Others	4 (9.5%)	6 (10.5%)	
Active smoking (n)			.787
No	40 (95.2%)	56 (98.2%)	
Yes	2 (4.8%)	1 (1.8%)	
Premarital health screening			.041
No	21 (50%)	17 (29.8%)	
Yes	21 (50%)	40 (70.2%)	
Pregnancy virus infection			.550
No	31 (73.8%)	45 (78.9%)	
Yes	11 (26.2%)	12 (21.1%)	
Passive smoke, h/day			.021
No	14 (33.3%)	26 (45.6%)	
<2	17 (40.5%)	21 (36.8%)	
2-3	5 (10.9%)	8 (14.0%)	
>3	6 (14.3%)	2 (3.6%)	
Heavy metals exposed			.022
Never	3 (7.2%)	10 (17.5%)	
Seldom	35 (83.3%)	47 (82.5%)	
Always	4 (9.5%)	0	
Plasticizer exposed			.829
No	13 (31.0%)	20 (35.1%)	
Yes	29 (69.0%)	37 (64.9%)	
Alcohol used			1.000
No	38 (90.5%)	52 (91.2%)	
Yes	4 (9.5%)	5 (8.7%)	
Take medicine during pregnancy			.378
No	27 (64.3%)	42 (73.7%)	
Yes	15 (35.7%)	15 (26.3%)	

* $P < 0.05$ There are differences between case group and the control group.

of Pb ($P = .038$) and Cd ($P = .010$) were significantly higher in the case group.

Furthermore, we were unable to detect DMP and DEP from the hair samples. The reason for this may be since these 2 kinds of phthalic acid ester in hair were under limit of detection (LOD). However, we successfully detected DBP, BBP, and DEHP from the 2 groups; no significant differences in DBP and BBP were observed ($P > .05$), while the significantly higher concentration of DEHP was observed in the case group ($P = .001$). At the 0 to 4 mg/L level of DEHP, the proportion of control group (91.23%) was higher than those of the cases (59.52%). But at the 4 to 6 mg/L level of DEHP, the proportion of case group (19.05%) was significantly higher than those of the controls (3.51%).

3.3. Potential factor exposure and risk of MA

Table 3 shows the logistic regression model that was used to estimate the potential factors associated with MA. In the initial analysis, statistically significant factors, that is, age, Pb, Cd, nicotine, cotinine, and DEHP were entered in the model. Age significantly increased the risk of MA ($P = .012$, OR 1.172, 95% CI 1.036–1.327). Pb exposure was significantly associated with an increased MA risk ($P = .070$, OR 8.26, 95% CI

Table 2
Distribution of smoking chemicals, heavy metal and phthalates in the hairs samples of 2 groups.

Chemicals	Case group (n=42)	Control group (n=57)	P*
Nicotine, μg/g†			.037
0–0.5	32 (76%)	53 (92.98%)	
>0.5	10 (23.81%)	4 (7.02%)	
Cotinine, μg/g†			.018
0–1	30 (71.43%)	50 (87.72%)	
>1	12 (28.57%)	7 (12.28%)	
Pb, μg/g†			.038
0–0.68	31 (73.81%)	52 (91.23%)	
>0.68	11 (26.19%)	5 (8.77%)	
Cd, ng/g†			.010
0–35	30 (71.43%)	52 (91.23%)	
>35	12 (28.57%)	5 (8.77%)	
Mn, μg/g			.137
0–1.90	34 (80.95%)	52 (91.23%)	
>1.90	8 (19.05%)	5 (8.77%)	
DBP, mg/L			.181
0–2	39 (92.86%)	56 (98.25%)	
2–4	3 (7.14%)	1 (1.75%)	
BBP, mg/L			.745
0–2	33 (78.57%)	46 (80.70%)	
2–4	7 (16.67%)	10 (17.54%)	
4–6	0	0	
>6	2 (4.76%)	1 (1.75%)	
DEHP, mg/L†			.001
0–2	14 (33.33%)	35 (61.40%)	
2–4	11 (26.19%)	17 (29.83%)	
4–6	8 (19.05%)	2 (3.51%)	
>6	9 (21.43%)	3 (5.26%)	

BBP=butyl benzyl phthalate, Cd=cadmium, DBP=dibutyl phthalate, DEHP=di-2-ethyl hexyl phthalate, Mn=manganese, Pb=plumbum.

* P value is for chi-square (numeration dates).

† Dates on these variables between on case group and control group are significantly different. P<0.05.

1.779–38.342). Cd exposure increased the risk of MA ($P = .040$, OR 8.931, 95% CI 2.003–39.811). Nicotine exposure was associated with an increased MA risk ($P = .090$, OR 8.347, 95% CI 1.707–40.418). Cotinine exposure was also a risk factor for MA ($P = .029$, OR 4.376, 95% CI 1.159–16.513). Compared to low DEHP content in the hair, high DEHP content increased the possibility of MA ($P = .020$, OR 1.863, 95% CI 1.103–3.146).

4. Discussion

According to the results of the present study, the statistically higher average age was observed in the case group (28.76) compared to the control group (26.7), suggesting the age is a risk factor for MA. This data reconfirmed findings from previous

Table 3
Model of logistic regression of potential factors on missed abortion.

Variable	Odds ratio	95% confidence interval	P
Age	1.172	1.036–1.327	.012
Pb	8.260	1.779–38.342	.070
Cd	8.931	2.003–39.811	.040
Nicotine	8.347	1.707–40.418	.090
Cotinine	4.376	1.159–16.513	.029
DEHP	1.863	1.103–3.146	.020

Cd=cadmium, DEHP=di-2-ethyl hexyl phthalate, Pb=plumbum.

studies,^[17–19] which demonstrated that age was strongly associated with MA occurrence. This might be because in older pregnancy ovaries, uteri are reduced, and the egg quality drops, causing chromosomal changes.^[18,19]

Studies have shown that,^[17–20] before getting pregnant, partner that smokes presents a risk factor for pregnant women abortion. Pregnant women exposure to tobacco smoke also increases the risk of placental abruption, placenta previa, fetal growth restriction, and other pregnancy complications.^[21] Active smokers inhale first hand smoke, while passive smokers inhale side stream smoke which due to the incomplete combustion of tobacco, contains harmful material more dangerous than the mainstream smoke.^[22] Tobacco smog effect in pregnant women is mainly due to harmful substances (such as nicotine, cotinine, carbon monoxide, and tar) in the smoke which cause pathological changes leading to the placenta calcification, which in turn includes shorter microvilli, reduced placental blood vessels, and increased collagen content of fuzzy matrix.^[23] It can furthermore increase the placenta and uterus contraction, reducing the flow of blood leading to miscarriage and stillbirth.^[13] Nicotine and cotinine from the mother's body may enter the placental circulation having direct toxic effect on the fetal cardiovascular system.^[24] In this study, the levels of nicotine OR=3.31, 95% CI: 0.01387–0.54547, and cotinine (OR=2.16, 95% CI: 0.51264–0.58607), in the hair of the case group were higher compared to normal pregnancy group, and the differences were statistically significant ($P < 0.05$). This implies that pregnant women early exposure to tobacco smoke environment increases the risk of MA.

Exposure to environmental pollution and occupational, heavy metal elements can translocate through the placenta from pregnant woman's body to the fetus where they accumulate.^[25] Erikson et al^[26] found that excessive exposure to Mn during pregnancy, in addition to inducing obvious neurotoxicity in pregnant women, it can also cause long-term growth retardation in offspring. Contrary, if pregnant women should experience serious lack of Mn, this might lead to fetal congenital malformations, stillbirth, or habitual abortion.^[27] Our research results showed no statistically significant difference between Mn levels in the hair of control group and case group, implying the occurrence of MA was not necessarily associated to hair Mn content. However, the levels of Pb, Cd in the hair of the case group were higher compared to controls. The toxicity of Pb on fetal embryo works through reduced membrane Na⁺, K⁺ atpase activity. Pb can inhibit ovarian corpus luteum cells synthetic progesterone key enzyme activity of steroid hormones; and can interfere with ovarian hormone stimulation on the lining of the uterus. Furthermore, Pb can cause endometrial atrophy, and make disintegrating uterine decidua cells release a large number of phosphatase. Transforming arachidonic acid to prostaglandins causes lining of the uterus to contract, which may be the main reason behind miscarriage and stillbirth.^[28] According to the European Economic Community (EEC) and the organization for economic co-operation and development (OECD) teratogenic content classification, Cd belongs to potential teratogenic. Cd contributes in developmental toxicity in animals.^[29] Cd suppresses the embryonic cells through the placenta and DNA replication and protein biosynthesis, especially on the nucleoli rRNA synthesis or significantly inhibited transcription. Existing research has shown that the accumulation of Cd can provoke morphological changes of the placenta, such as increased maternal fetal capillaries gap, with smaller diameter. Cd may in turn affect the blood supply of the placenta and fetal blood

flow.^[27] Pb, Cd may also inhibit the biosynthesis of embryonic cells and damage the structure of the cell membrane, in turn causing pathological changes, and miscarriage.

We detected 5 phthalates (DMP, DEP, DBP, BBP, and DEHP) in the hair of pregnant women. Besides the concentration of DMP and DEP that were below LOD, we could detect DBP, BBP and DEHP of all participants. This means that a lot of pregnant women had no knowledge about phthalate exposure and were probably not taking any precautions to avoid these chemicals. According to our research, the levels of DBP and BBP were high but without statistical significance in the 2 groups. However, the level of DEHP in the case group was significantly higher compared to the controls. In lower level of DEHP, there were no differences between the 2 groups. But in the higher level, there were significant differences between the 2 groups. The obtained results were similar with the dose–response relationships between urinary phthalate concentrations and risk of clinical pregnancy loss.^[30] Our results showed that value OR for DEHP was 1.83, suggest that the exposure to high level of DEHP may be a risk factor for MA. Adverse female reproductive and developmental outcomes have been observed in several animal studies where the exposure to specific phthalates [DEP, DEHP, di-*n*-butyl phthalate (DnBP), and di-iso-butyl phthalate (DiBP)] were linked with decreased embryo survival, increased incidence of resorptions, reduced number and size of litters, and increased abortion rates in rats.^[31,32] Schmidt reported that chronic occupational exposure to high levels of phthalates has been linked with decreased rates of pregnancy and higher rates of miscarriages in female factory workers.^[33]

The present study has some limitations. Firstly, we did not measure the levels of human chorionic gonadotropin (hCG) in pregnant women, since once MA occurs the levels of hCG must decline. If we wanted to find the relation between MA and the levels of hCG, we should detect the levels of hCG before the embryo die, which is impossible in a case-control design. Secondly, due to a limited number in this group, the estimated OR was associated with a wide CI. If the sample size was larger, the results would be more convincing. Finally, this study did not test for chemicals in the blood or urine to confirm the results of the experiments.

In summary, our results suggest that high gestational age, passive smoking, heavy metals and the phthalate exposure are risk factors for MA. Premarital health examination is a strong protective factor against MA.

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