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Levels of PAH-DNA Adducts in Cord Blood and Cord Tissue and the Risk of Fetal Neural Tube Defects in a Chinese Population

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Abstract

Introduction—Maternal exposure to polycyclic aromatic hydrocarbons (PAHs) has been shown to be associated with an elevated risk for neural tube defects (NTDs). In the human body, PAHs are bioactivated and the resultant reactive epoxides can covalently bind to DNA to form PAH-DNA adducts, which may, in turn, cause transcription errors, changes in gene expression or altered patterns of apoptosis. During critical developmental phases, these changes can result in abnormal morphogenesis.

Objectives—We aimed to examine the relationship between the levels of PAH-DNA adducts in cord blood and cord tissue and the risk of NTDs.

Methods—From 2010 to 2012, sixty NTD cases and 60 healthy controls were recruited from a population-based birth defects surveillance system in five counties of Shanxi Province in Northern China, where the emission of PAHs remains one of the highest in the country and PAHs exposure is highly prevalent. PAH-DNA adducts in cord blood of 15 NTD cases and 15 control infants, and in cord tissue of 60 NTD cases and 60 control infants were measured using the ³²P-postlabeling method.

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Conflict of interest

The authors declare they have no competing financial or nonfinancial interests.

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Results—PAH-DNA adduct levels in cord blood tend to be higher in the NTD group (28.5 per 10^8 nucleotides) compared with controls (19.7 per 10^8 nucleotides), although the difference was not statistically significant ($P=0.377$). PAH-DNA adducts in cord tissue were significantly higher in the NTD group (24.6 per 10^6 nucleotides) than in the control group (15.3 per 10^6 nucleotides), $P=0.010$. A positive dose-response relationship was found between levels of PAH-DNA adducts in cord tissue and the risk of NTDs ($P=0.009$). When the lowest tertile was used as the referent and potential confounding factors were adjusted for, a 1.03-fold (95% CI, 0.37–2.89) and 2.96-fold (95% CI, 1.16–7.58) increase in the risk of NTDs was observed for fetuses whose cord tissue PAH-DNA adduct levels were in the second and highest tertile, respectively.

Conclusions—High levels of PAH-DNA adducts in fetal tissues were associated with increased risks of NTDs.

Keywords

Neural tube defects (NTDs); Polycyclic aromatic hydrocarbons (PAHs); DNA adducts; Cord tissue; Cord blood; Fetus

1. Introduction

Neural tube defects (NTDs) are serious life threatening birth defects affecting the brain and spinal cord, resulting from the failure of the embryonic neural tube to close between 18 and 28 days of gestation (Blom et al., 2006, Botto et al., 1999). NTDs rank among the most common and debilitating of human congenital abnormalities. It has been estimated that more than 320,000 infants are born with NTDs annually worldwide, with an average of one in every 1000 established pregnancies (Copp et al., 2013). The most common types of NTDs include anencephaly and spina bifida (meningomyelocele) (Botto, Moore, 1999). Most fetuses with anencephaly, characterized by the absence of the cranial vault and absent or markedly diminished cerebral hemispheres, are aborted or stillborn. While the majority of fetuses with spina bifida, characterized by meninges and spinal cord tissue that are exposed to the body surface are live born, those that survive suffer with a range of challenging life-long disabilities (Christianson AL, 2006). Despite decades of intensive study, the exact etiology of these congenital defects remains poorly understood. It is generally agreed that most NTDs represent a multifactorial disorder, arising from a complex combination of genetic and environmental interactions (Wallingford et al., 2013).

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous carcinogenic and teratogenic environmental pollutants resulting from incomplete combustion of fossil fuel and biomass that are commonly found in tobacco smoke, ambient and indoor air, and charbroiled foods. In laboratory animals, embryos exposed to derivatives of PAHs have been shown to present with NTDs (Barbieri et al., 1986, Incardona et al., 2004). Human studies have also indicated that maternal prenatal exposure to PAHs was associated with an elevated risk for NTDs in offspring, using residence location or biomarkers of exposure (Demetriou et al., 2012, Langlois et al., 2012, Rankin et al., 2009). Previously we demonstrated that indoor air pollution from coal combustion was a potential risk factor for NTDs in Shanxi Province, where the concentration of PAHs emissions is amongst the highest in the country. Furthermore, almost all rural households use coal for their residential heating and cooking

needs, resulting in further PAHs exposure in this population (Li et al., 2011). In the human body, PAHs are bioactivated and the resultant reactive epoxides can covalently bind to macromolecules including DNA to form PAH-DNA adducts (Whyatt et al., 1998). PAH-DNA adducts, widely used as biomarker of biologically effective dose from all sources of PAH exposure, can reflect individual variation in exposure, absorption, metabolic activation, and DNA repair capabilities (Demetriou, Raaschou-Nielsen, 2012). Formation of PAH-DNA adducts increases the risk of genotoxic effects and leads to carcinogenesis (Roshandel et al., 2012, Veglia et al., 2008) and other potentially adverse outcomes.

PAHs and their metabolites can readily cross the placental barrier to reach the embryo, which may be 10 times more susceptible than the mother to PAH-induced DNA damage (Perera et al., 2005). A variety of adverse birth outcomes such as: neurodevelopment deficits (Perera et al., 2008), impaired fetal growth (Perera, Tang, 2005, Sram et al., 2006), and aberrant child behavior (Perera et al., 2011) have been shown to be associated with elevated PAH-DNA adduct levels in cord blood. To our knowledge, the level of PAH-DNA adducts in NTD affected fetuses and the association with the risk of NTDs has not been previously reported. Although an inverse association between levels of PAH-DNA adducts in maternal venous blood and placental tissue and the risk of NTDs was observed in our previous studies (Naufal et al., 2010, Yuan et al., 2013), the maternal venous blood and placenta might not be good surrogates for adduct formation in fetal organs. In this study, we sought to examine whether the levels of PAH-DNA adducts in cord blood or cord tissue were associated with the risk of NTDs in the exposed offspring.

2. Materials and methods

2.1. Subjects and sampling

The study design has been described in our previous reports (Li et al., 2007, Ren et al., 2011). Briefly, subjects were recruited from a population-based birth defects surveillance system in five rural counties of Shanxi Province (Taigu, Pingding, Xiyang, Shouyang and Zezhou) in northern China between 2010 and 2012. Cases were fetuses or newborns with a confirmed diagnosis of a NTD, while controls were healthy newborn infants with no congenital malformations. Once a case was identified, a control born in the same hospital was selected, matched to the case by sex, mother's county of residence, and the date of mother's last menstrual period, which was selected to be as close as possible to that of the case mother. The interview participation rate was about 80% for both case and control mothers. Information on the mother's socio-demographic characteristics, lifestyle, reproductive history, periconceptional use of folic acid supplements, smoking and passive smoking, and exposure to domestic fuel use for cooking and heating was collected through face-to-face interviews. Umbilical cord and whole cord blood was collected at the time of delivery or termination of NTD-affected pregnancies. Umbilical cord was placed in polyethylene bags, and immediately stored at -20°C until used for analyses. Cord blood was obtained in EDTA collection tubes, and centrifuged to separate blood cells within 4 h of blood collection. Blood cells were transferred by pipette to a plastic freezing tube which was stored at -80°C for future DNA isolation and analysis. In this study, we randomly selected 60 umbilical cord tissue samples of NTD cases (anencephaly $n=22$; spina bifida $n=38$)

from a total of 97 NTD-affected fetuses/newborns, and 60 control samples from 276 umbilical cords of healthy infants. Fifteen cord blood samples were available from the above 60 NTD cases, and 15 cord blood samples were randomly selected from the 60 healthy control infants. This study was conducted according to the guidelines established in the Declaration of Helsinki, and all procedures involving human subjects were approved by the institutional review board of Peking University, and written informed consent was obtained from all the mothers participating in the study.

2.2. Laboratory analyses

2.2.1. DNA isolation—QIAamp DNA Blood Mini Kit from QIAGEN (Cat no.51104) was used to extract DNA from cord blood cells following the manufacturer's protocols. DNA was extracted from umbilical cord tissue using a standard procedure. Briefly, one section of the umbilical cord (about 1 g) including the Wharton's jelly and blood vessels, was dissected free and subsequently minced. The umbilical cord tissue was washed manually with ice-cold PBS (pH 7.0) to remove any residual blood. The tissue pellet was homogenized with a solution of PBS and centrifuged. DNA was isolated using RNase A and proteinase K treatment, followed by phenol/chloroform/isoamyl extraction as described previously (Yuan, Jin, 2013). DNA concentrations and purity were determined by measuring the spectrophotometric absorbance at 260 and 280 nm and the A_{260}/A_{280} ratio for all samples was between 1.8 and 2.0.

2.2.2. ^{32}P -Postlabeling—PAH-DNA adducts in umbilical cord and cord blood were determined by the nuclease P1 enhanced ^{32}P -Postlabeling method, which is highly sensitive and can be used to detect various DNA adducts with multiple structures (Phillips and Arlt, 2007). The protocol of the ^{32}P -postlabeling method has also been previously described (Yuan, Jin, 2013). Briefly, an aliquot (5 μg) of each DNA sample was degraded enzymatically to mononucleotides. After 3'-dephosphorylation of the normal nucleotides with nuclease P1, the enriched nuclease P1-resistant modified 3'-nucleotides were converted to 5'- ^{32}P -labeled deoxyribonucleoside 3',5'-bisphosphate derivatives by incubation with carrier-free [γ - ^{32}P]ATP and T4 polynucleotide kinase. The labeled adducts were separated by multidirectional thin-layer chromatography. Profiles and levels of DNA adducts were detected and quantified by phosphorimage analysis (Typhoon Trio, GE Healthcare). For each batch of assay, a positive control consisting of BaP-diolepoxide DNA adduct was also analyzed. The calculated DNA adducts of positive controls for each assay was maintained within the range of 90%-110% of the standard. Fig.1 shows the typical profiles of DNA adducts from cord tissue (a) and cord blood (b). DNA samples from cases or controls exhibited qualitatively similar profiles. A positive control sample obtained from calf thymus DNA treated with B[a]P displayed similar pattern of adducts (c), which we have previously described (Yuan, Jin, 2013). Adducts 1–2 were summed up as total PAH-DNA adducts for further analyses. DNA adduct levels were quantified and expressed as adducts per 10^6 nucleotides for umbilical cord, and per 10^8 nucleotides for cord blood.

2.3. Statistical analysis

The median with interquartile range was used to describe the distribution of adduct levels, which were not normally distributed. Median of adduct levels between groups were

compared by Mann-Whitney U test. In dose-response analysis, tertile of total PAH-DNA adducts in controls was used as cutoff values. Risk of NTDs associated with PAH-DNA adduct levels was estimated from the odds ratio (OR) with 95% confidence interval (CI) by unconditional logistic model. Maternal age, educational level, occupation, parity, obesity, periconceptional folate supplement, hyperthermia during pregnancy were selected as potential confounding factors on the basis of their possible relationship with both the PAH exposure and an increased risk for NTDs, and were adjusted in the multivariate model. Statistical analyses were conducted using SPSS 20.0. A two-tailed *P* value of 0.05 was considered to indicate statistical significance.

3. Results

3.1. Characteristics of the subjects

The study consisted of 60 NTDs cases (22 anencephalic and 38 spina bifida) and 60 unaffected controls. Distribution of maternal and fetal characteristics was summarized in Table 1. There were no significant differences between the two groups with regard to maternal age, occupation, parity, folic acid supplementation, obesity, primary fuel used for cooking and fetal sex. NTD case mothers had higher proportion of hyperthermia during early pregnancy, maternal passive smoking and coal used for heating, when compared to control mothers.

3.2. Levels of PAH-DNA adducts between the case and the control group

As shown in Table 2, the median level of adducts in case umbilical cords was 24.62 adducts/ 10^6 nucleotides, 61% higher than the level of 15.32 adducts/ 10^6 nucleotides observed in the control umbilical cords ($P=0.010$). Anencephalic cases (23.85 adducts/ 10^6 nucleotides) also showed higher adduct levels than controls ($P=0.010$), while spina bifida cases (24.60 adducts/ 10^6 nucleotides) tended to have higher levels of adducts than did controls ($P=0.060$). The median level of adducts in case cord blood were 28.50 adducts/ 10^8 nucleotides, which was 44% higher than the level of 19.74 adducts/ 10^8 nucleotides in control cord blood, but the difference did not reach statistical significance ($P=0.377$), likely due to limited sample size.

The association between higher levels of PAH-DNA adducts in umbilical cord tissue with an increased risk of NTDs showed a dose-response relationship (Table 3). When the lowest tertile was used as the referent, 1.03-fold (95% CI, 0.37–2.89) and 2.96-fold (95% CI, 1.16–7.58) increases in the risk of NTDs were observed for fetuses whose umbilical cord concentrations of PAH-DNA adducts were in the second and highest tertile, respectively. Trend analysis showed a statistically significant linear trend for the associated risks ($P_{\text{trend}}=0.009$). A dose-response relationship was also present for spina bifida and anencephaly subtype ($P_{\text{trend}}=0.035$ and 0.028 respectively). A 2.97-fold (95% CI, 1.18–7.49) and 3.12-fold (95% CI, 1.14–8.55) increases in the risk of spina bifida and anencephaly were observed for fetuses who had the highest tertile of PAH-DNA adducts in their cord tissue.

4. Discussion

In this study, we investigated the levels of PAH-DNA adducts in cord blood and cord tissue and the risk of NTDs in infants from Shanxi Province in northern China. We found that NTD risk increased with increasing PAH-DNA adduct level in the cord tissue. To our knowledge, this is the first study to identify a positive association between PAH-DNA adducts in the fetus and increased risk of NTDs.

Maternal prenatal exposure to PAHs has been found to be a risk factor for fetal NTDs and other congenital malformations in several previously published epidemiological studies. Maternal occupational exposure to PAHs was associated with increased risks of congenital heart defects (Lupo et al., 2012b), gastroschisis (Lupo et al., 2012a) and NTDs (Langlois, Hoyt, 2012). In our previous study, maternal passive smoking and exposure to indoor coal combustion had been shown to be associated with an elevated NTD risk. In the present study, an association between maternal passive smoking and use of coal for residential heating and increased risk of NTDs were also observed (Li, Zhang, 2011). In the rural area of Shanxi Province, indoor coal combustion is one of the major sources of exposure to PAHs in addition to industrial emission, because local residents use coal for cooking and residential heating in the winter. Our previous studies in Shanxi Province also found that the levels of PAHs in the venous blood and placenta tissues from mothers of NTD-affected fetuses were higher than in those of mothers of healthy infants (Naufal, Li, 2010, Ren, Qiu, 2011). PAHs have been consistently shown to be teratogenic in animal models. NTDs and a variety of other congenital malformations were induced by PAHs in mouse embryos (Barbieri, Ognio, 1986), and the development of neural tube structures was disturbed in PAH exposed fish embryos (Incardona, Collier, 2004).

PAHs can readily pass through placenta and exert genotoxic effects on the developing fetus through metabolic activation and subsequently forming bulky PAH-DNA adducts (Neubert and Tapken, 1988, Perera, Tang, 2005). The amount of PAH-DNA adducts in infant cord blood was positively associated with prenatal exposure to PAHs (Jedrychowski et al., 2013, Pedersen et al., 2009, Whyatt, Bell, 1998). Fetal DNA is prone to more DNA damage and the adduct levels in the newborns were similar to or higher than that observed in the mothers, despite the transplacental dose of PAH in the fetus estimated to be 10 percent of that found in paired maternal tissues (Perera, Tang, 2005, Whyatt et al., 2001). Animals experiments have revealed increased susceptibility to DNA damage may contribute to the greater carcinogenic effect of PAHs. In the present study, PAH-DNA adduct levels in cord blood tended to be higher in the NTDs group than in controls. The lack of statistical significance is apparently due to the small sample size (cases/controls=15/15), which is underpowered ($1-\beta=0.24$) to detect any differences. Most of the NTD-affected fetuses were aborted between 12–20 weeks of gestation; therefore the cord blood was not available for most NTD cases. The umbilical cord is physiologically and genetically part of the fetus, which develops from the yolk sac and allantois, therefore umbilical cord tissue may be used as a good biological surrogate to measure the levels of PAH-DNA adducts in the fetus. It is estimated that cord blood PAH-DNA adducts levels may be able to reflect past exposure of 4 months (Jedrychowski, Perera, 2013). However, there is no data on the half-life of PAH-DNA adducts in fetal tissue in the literature, to the best of our knowledge. It is reasonable to

assume that the half-life of PAH-DNA in tissue is longer than cord blood due to the longer turn over of the former than the latter. This makes cord tissue the biospecimen that most relevant in terms of exposure window and neural tube development, in addition to the easy accessibility of the cord tissue in human studies.

Our previous preliminary studies have found that mothers with NTD-affected fetuses had higher PAH concentrations but lower PAH-DNA adduct levels in maternal venous blood as well as in placental tissue, than did mothers of healthy infants (Naufal, Li, 2010, Yuan, Jin, 2013). There is no ready explanation for these results that seemingly contradict to the present findings. Perhaps, mothers of NTD cases may have a lower metabolic rate and therefore facilitate increased PAH translocation across the placental barrier to reach the fetus, resulting in the higher levels of PAH-DNA adducts detected in fetuses with NTDs. Human and experimental evidence indicates that the developing fetus is especially vulnerable and more susceptible to chemical carcinogens, including PAHs, tobacco smoke, and air pollution, compared with adults (Perera et al., 2004, Whyatt, Jedrychowski, 2001). The developing embryos may be as much as 10 times more susceptible than the mother to PAH-induced DNA damage (Perera, Tang, 2005). It is thought that the fetus could have greater retention of PAHs, lower immunologic competence, weaker detoxification and DNA repair capacity, all of which could cause developmental deficits given the increased rate of cell proliferation during early stages of development (Anderson et al., 2000). A few prior studies have reported that neurodevelopment deficits (Perera, Li, 2008) and impaired fetal growth (Perera, Tang, 2005, Tang et al., 2006) were associated with high PAH-DNA adduct levels in cord blood.

The mechanisms underlying the association between PAH-DNA adducts and the failure of neural tube closure are not fully appreciated. Studies have shown that the formation of DNA adducts induced by PAHs can increase Ser-15 phosphorylation of p53 tumor suppressor levels, which is associated with the induction of the apoptotic pathways (Nicol et al., 1995, Topinka et al., 2008). Animal studies have indicated that NTDs induced by *in utero* valproic acid exposure was mediated by apoptosis (Mallela and Hrubec, 2012, Tung and Winn, 2011). PAH exposure has also been associated with epigenetic alterations. B[a]P-DNA adduct formation has been shown to affect DNA methylation patterns in experimental systems (Sadikovic et al., 2007, Wilson and Jones, 1983). Early embryogenesis could be a particularly susceptible period for epigenetic dysregulation as a consequence of environmental exposures, as DNA methylation is epigenetically reprogrammed (Dolinoy et al., 2007). Additionally, epigenetic alterations have emerged as an important mechanism involved in the complex etiology of NTDs (Dunlevy et al., 2006, Liu et al., 2012, Wang et al., 2010). Further studies are needed to investigate the underlying mechanisms linking PAH-DNA adducts to increased NTD risk.

There are several strengths in the present study. First, our collected individual prenatal exposure data from questionnaire data was extensive, providing us with information on potentially important confounding factors of NTD risk such as: maternal age, educational level, occupation, parity, obesity, periconceptional folate supplementation, and hyperthermia during early pregnancy. In addition, we were able to quantitatively measure the individual biologically effective dose of PAHs through the measurement of PAH-DNA adducts by ³²P-

Postlabeling method. The primary limitation of this study is that the cord tissue was sampled at different gestational stages among NTD cases and controls. This drawback could not be eliminated with routine case-control design, as the umbilical cord of healthy controls can only be sampled at term. However, we found that the levels of PAH-DNA adducts were not associated with the gestational age in the NTDs group ($P=0.425$). Another limitation is related to the time window-the development of NTDs occurred early in gestation, yet the cord tissue was sampled later in pregnancy. However, the continuum of development from the yolk sac and allantois to the umbilical cord and a relatively long period of past exposure that PAH-DNA adducts could reflect (Jedrychowski, Perera, 2013, Mooney et al., 1995) make cord tissue a reasonable surrogate organ of the fetus in which PAH exposures that occurred earlier in gestation can be evaluated. A third limitation is the relative sample size, which limited the power to detect a difference in cord blood PAH-DNA adducts and in folic acid supplementation between the case and the control groups.

5. Conclusion

We found that higher levels of PAH-DNA adducts in fetal body were associated with increased risks of NTDs. Further studies are warranted in other human populations to replicate these findings and animal experiments are needed to reveal the mechanisms underlying the association between PAH-adducts and fetal NTD risk. If these are confirmed, the results presented here have implications for risk assessment and environmental health policy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights of this research

1. PAH-DNA adducts in cord tissue and cord blood of neural tube defects affected fetuses and healthy infants were detected.
2. We used the ^{32}P -postlabeling method to detect the PAH-DNA adducts levels.
3. Subjects were recruited from a population-based birth defects surveillance system in Shanxi Province, where the concentration of PAHs emissions is amongst the highest in the country.
4. High levels of PAH-DNA adducts in fetal tissues were associated with increased risks of neural tube defects.

Fig.1.

Profile of DNA adducts from cord tissue (a), cord blood (b) and calf thymus DNA treated with B[a]P (c). Images were from a phosphoimager (Typhoon Trio, GE Healthcare) after exposing to a storage phosphor screen for 2 h.

Table 1

Demographic and Obstetric Characteristics of Neural Tube Defect Cases and Controls in a Chinese population, 2010–2012

Characteristic	Cases (n=60) ^a	Controls (n=60) ^a	P value ^b
Maternal age (y)			0.287
<25	20 (33.3)	28 (46.7)	
25–30	20 (33.3)	18 (30.0)	
>30	20 (33.4)	14 (23.3)	
Maternal education			0.015
Primary or lower	6 (10.0)	6 (10.0)	
Junior	47 (78.3)	34 (56.7)	
High	7 (11.7)	20 (33.3)	
Occupation			0.345
Farmer	51 (85.0)	47 (78.3)	
Non-farmer	9 (15.0)	13 (21.7)	
Obesity			0.378
No	52 (86.7)	55 (91.7)	
yes	8 (13.3)	5 (8.3)	
Parity			0.224
1	27 (45.0)	34 (56.7)	
2	33 (55.0)	26 (43.3)	
Periconceptional folate supplementation			0.463
No	35 (58.3)	31 (51.7)	
Yes	25 (41.7)	29 (48.3)	
Hyperthermia during pregnancy			0.022
No	43 (71.7)	53 (88.3)	
Yes	17 (28.3)	7 (11.7)	
Maternal passive smoking			<0.001
No	37 (61.7)	57 (95.0)	
Yes	23 (38.3)	3 (5.0)	
Primary fuel used for cooking			0.136
Coal	40 (66.7)	32 (53.3)	
Natural gas/other	20 (33.3)	28 (46.7)	
Primary fuel used for heating			0.005
Coal	30 (50.0)	15 (25.0)	
Natural gas/other	30 (50.0)	45 (75.0)	
Gestational age (weeks)			<0.001
<28	32 (53.3)	-	
28–36	15 (25.0)	-	
>36	13 (21.7)	60 (100)	
Fetus sex			0.855
Male	28 (46.7)	27 (45.0)	

Characteristic	Cases (n=60) ^a	Controls (n=60) ^a	P value ^b
Female	32 (53.3)	33 (55.0)	

^aData are presented in number (percentage).

^bCases and controls are compared by Pearson's chi-square test.

Table 2

Levels of PAH-DNA adducts in the umbilical cord tissue and cord blood of cases with neural tube defects (NTDs) and healthy control newborns in a Chinese population, 2010–2012.

Characteristic	N	Median	Interquartile range	<i>P</i> value ^b
Umbilical cord tissue ^a				
Total NTDs	60	24.62	11.62–31.67	0.010
Spina bifida	38	24.60	10.27–30.89	0.060
Anencephaly	22	23.85	12.93–41.83	0.016
Controls	60	15.32	7.37–25.03	
Cord blood ^a				
Total NTDs	15	28.50	15.23–68.59	0.377
Controls	15	19.74	14.74–29.90	

^aConcentration of PAH-DNA adducts in the umbilical cord tissue and cord blood were expressed as per 10⁶ nucleotides and per 10⁸ nucleotides, respectively.

^bIn comparison with the median of controls, Mann-Whiney U test.

Multivariate analysis on the association between tertiles of PAH-DNA adducts in umbilical cord tissue and the risk of neural tube defects (NTDs) in a Chinese population, 2010–2012.

Table 3

Adduct level ^a	Control		Total NTDs		Spina bifida		Anencephaly	
	N		N	OR (95% CI) ^b	N	OR (95% CI) ^b	N	OR (95% CI) ^b
1st tertile	21	13	9	1.0 (ref.)	4	1.0 (ref.)		
2nd tertile	20	13	8	1.03 (0.37–2.89)	9	0.6 (0.19–2.52)	5	1.43 (0.33–6.24)
3rd tertile	19	34	21	2.96 (1.16–7.58)	7	2.9 (1.18–7.49)	13	3.12 (1.14–8.55)
P for trend				0.009		0.035		0.028

^aTertile of total PAH-DNA adducts in controls was used as cutoff values.

^bMaternal age, educational level, occupation, parity, hyperthermia during pregnancy, obesity, periconceptional folate supplement were adjusted for.