Int J Biochem Mol Biol 2013;4(4):209-214
www.ijbmb.org /ISSN:2152-4114/IJBMB1310004

Original Article

Gene environment interaction in preterm delivery with special reference to organochlorine pesticide: a case control study

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Received November 13, 2013; Accepted November 28, 2013; Epub December 15, 2013; Published December 30, 2013

Abstract: Objectives: To assess the Gene-Environmental interaction between maternal organochlorine pesticides (OCPs) level and *CYP17* gene polymorphism with the risk of preterm delivery (PTD). Materials and methods: Maternal blood samples of hundred cases (n = 100) of PTD and of equal number of healthy controls were collected at the time of delivery. OCPs levels were estimated by Gas chromatography system equipped with electron capture detector and PCR-RFLP was used for polymorphic analysis of *CYP17* gene. Results: Significantly ($p < 0.05$) higher levels of α-HCH, β-HCH, and γ-HCH were found in maternal blood samples of PTD cases as compared to controls. We did not found any significant difference in the frequency genotype distribution CYP17 gene in PTD cases as compared to controls. When gene environmental interaction between the *CYP17* gene polymorphism and OCPs level was considered, a significant interaction was observed between ≥ 50th percentile of γ-HCH and *CYP17* A1A1 (wild type) genotype. Conclusions: Higher levels of OCPs along with wild type state of *CYP17* gene (A1A1) in women may be considered as an important etiological factor in 'idiopathic' PTD. The present study provides evidence that genetic variation and its interaction with the environmental exposure may increase the risk of PTD.

Keywords: Preterm delivery, organochlorine pesticides, *CYP17* gene polymorphism, gene-environment interaction

Introduction

Preterm delivery (PTD), period of gestation < 37 weeks, is the largest cause of prenatal deaths, neonatal morbidities, mortality, and adult illness [1]. According to a WHO report, India tops the list of countries; with maximum number of preterm deliveries (WHO report Born too soon 2012) PTD is among the top causes of death in infants worldwide. The exact cause of preterm delivery is also unsolved. In fact, the cause of 45-50% of preterm deliverys is never determined [2]. Labor is a complex process involving many factors. Four different pathways have been identified that can result in preterm delivery and have considerable evidence: precocious fetal endocrine activation, uterine overdistension, decidual bleeding, and intrauterine inflammation/infection [3]. Studies, including those from our laboratory have shown that environmental pollutant such as organochlorine pesticides (OCPs) may lead to preterm delivery [4-7].

OCPs have been widely used in India for agriculture and public health programs [8]. OCPs are xenoestrogenic in nature, are chemically stable and strongly lipophilic, and have a long half life [9]. The OCPs are more harmful in the females because of their higher percentage of body fat [10]. Cytochrome P450c17α enzyme encoded by *CYP17* gene functions at key steps in the estrogen synthesis pathway. *CYP17* regulates cortisol biosynthesis and potentially induce preterm delivery [11]. Although environmental factors are important, genetic factors also play a very important role in the preterm delivery. Gene environmental models have explained why every woman exposed to environmental contaminants does not have adverse reproduc-

tive outcomes. Recent study, from our laboratory has shown that the Gene environment interaction of *GSTMI/GSTT1* gene with OCPs decreased the period of gestation [2]. No study so far has determined the OCP levels and its association with CYP17 polymorphs in North population. Hence, the present study was aimed to identify the risk posed by OCPs in preterm delivery and its association with genetic polymorphism in CYP17 gene.

This study will attempt to identify the gene-environment interaction between CYP17 and OCPs residue levels which may improve our understanding of preterm delivery and provide new therapeutic targets in future.

Materials and methods

Participant recruitment and collection of samples

The present study was a hospital-based case control study. A total number of hundred (n = 100) women delivering PTD babies (case) and an equal number of women delivering healthy term babies (control) were included in this study after their admission to Guru Teg Bahadur Hospital, Delhi. A questionnaire survey of the women was done to collect general demographical information in order to define the inclusion/exclusion criteria. We excluded potentially confounding factors such as women with occupational exposure to pesticides and farming communities from this study. Women with only general environmental exposure were included, and pesticides detected were from contamination of the food chain and/or environmental pollution. Consent was obtained from all the study participants before collection of the sample and commencement of the study. The study design was duly approved by the Institutional Ethical Clearance Committee for Human Research. Three-milliliter (ml) samples, were collected in an EDTA-containing vial and stored at 4 °C until further analysis. Two ml maternal and cord blood was used for OCP analysis and 1 ml of maternal blood for DNA isolation. Genomic DNA was stored at -20 °C until genotyping.

Extraction of OCPs from blood and clean-up of the samples

All the solvents used for OCP extraction were of HPLC-grade and free from any contamination. OCPs extraction was done using hexane and acetone (2:1) according to previously described method [2] with minor modifications. Hexane and acetone (in ratio of 2:1) were added to blood and the whole mixture was shaken at room temp for 30 min in a mechanical shaker. The extract was centrifuged at 2000 rpm for 10 min and clear top layer of hexane was collected. The remaining portion was again extracted twice using same process and the newly extracted hexane layer was added to the previous solvent fractions. Clean-up of the samples was done by column chromatography following USEPA method 3620B. Elute was collected, concentrated and re-dissolved in hexane for further analysis. Quantification of OCP levels was done by Perkin Elmer Gas Chromatograph (GC) equipped with 63Ni selective Electron Capture Detector. The column used was Elite-GC DB-5, 60 meter and 0.25 mm ID. The carrier and makeup gas was nitrogen with a flow rate of 2 mL min^{-1} and 35 mL min^{-1} respectively, employing the split less mode. Final extract (1 µL) was injected at a temperature of 170 °C with a hold time of 1 min. The temperature was raised from 170 °C to 225 °C at a rate of 5 °C min^{-1} with a hold time of 5 min and finally from 225 °C to 275 °C at a rate of 6 °C min^{-1} with a hold time of 15 min. The total run time was 40 min per sample.

Quantitative analysis of OCP residues of each sample was done by comparing the peak area with those obtained from a chromatogram of a mixed OCPs standard (Supelco, Sigma-Aldrich) of known concentration. Analytes were confirmed by spiking with known standards of pesticides (Supelco, Sigma-Aldrich). The detection limit of the detector was 0.05 pg perchloroethylene with nitrogen as a carrier gas. The detection limit of the method was 4 pg mL^{-1} for each OCP. For quality control, five blood samples in triplicate were spiked with a mixed standard of OCPs at 5 and 25 ng mL^{-1}. The average recoveries of fortified samples exceeded 95%. The case and control samples were run in the same analytical batches and for quality check, a sample was always run with each set of samples for pesticide analysis to maintain accuracy.

Genomic analysis

Genomic DNA was extracted by using commercially available Himedia Hipura DNA isolation kit (Mumbai, India), as per manufacturer's protocol. Polymorphic analysis of the *CYP17* gene was carried out by PCR- restriction fragments length polymorphism (PCR-RFLP). Genotyping

Table 1. Demographical characteristics of women with PTD (cases) and FTD (controls)

Characteristics	FTD (n = 100) (Mean ± S.D)	PTD (n = 100) (Mean ± S.D)	*p* value
Maternal age	24.20±2.91	23.50±2.50	0.002*
Gestational age	39.03±1.07	32.91±1.72	0.001*
Baby weight	3.00±0.566	2.70±0.513	0.001*
Maternal Education			0.001*
Illiterate	8 (8%)	18 (18%)	
Literate	92 (92%)	82 (82%)	
Drinking water			0.174
Govt. sources	92 (92%)	88 (88%)	
Private source	8 (8%)	12 (12%)	
Residential area			0.212
Urban	97 (97%)	95 (95%)	
Rural	3 (3%)	5 (5%)	
Food habit			0.404
Vegetarian	62 (62%)	59 (59%)	
Non vegetarian	38 (38%)	41 (41%)	
Socioeconomic status			0.649
Class I (Upper)	5 (5%)	3 (3%)	
Class III (Middle)	60 (60%)	62 (62%)	
Class V (Lower)	35 (35%)	37 (37%)	

*Unpaired 't' test was applied for quantitative variables like age, period of gestation, baby weight etc., Chi-square/Fisher's exact test for qualitative data like area, food habits, source of drinking water etc. p < 0.05 is significant Figure in parenthesis indicate percent values.

of the polymorphism in the 5'-untranslated region of the *CYP17* gene was determined by PCR-RFLP analysis using restriction enzyme MspA1I (NEB, Ipswich, MA, USA) as per manufacturer's protocol. An aliquot of 100 ng of DNA was mixed with 0.5 mmol/leach of the primers (forward, 5'-TCCTGAGCCCAGATACCAT-3'; reverse, 5'-CCGCCCAGAGAAGTCCT-3'), 1.25 IU of Taq polymerase with 3.3 mmol/l $MgCl_2$ and 200 mmol/l dNTPs in a total volume of 50 µL of PCR buffer. The PCR products were digested with the restriction enzyme, separated by 2% agarose gel electrophoresis and identified with ethidium bromide staining. All the three possible genotypes for the polymorphism: A1A1 (homozygous wild-type), A1A2 (heterozygous variant type) and A2A2 (homozygous variant type) were detected.

Statistical analysis

To compare the quantitative value of demographic factors like age, period of gestation, neonatal anthropometric measurements unpaired 't' test was used, and for qualitative data like living style, food habits, socioeconomic status, parity and source of drinking water Chi-square/Fisher's exact test was used. Unpaired 't' test was used to compare the pesticide levels in cases and controls. Binary logistic regression was used to find an association between the genotypes of the *CYP17* gene. Taking as the dependent variable, multiple linear regression analysis was used to find any correlation of gene polymorphism and pesticide levels.

Results

The women who participated in the study were well matched for their demographic characteristics such as age, period of gestation, source of water supply, dietary habit etc. PTD was significantly ($p < 0.05$) associated with maternal age, gestation period, baby weight and maternal education (**Table 1**). The polymorphic variants of the *CYP17* gene were assessed in the maternal DNA to examine if the polymorphism provided any causative role in the pathophysiology of PTD. The frequency of wild genotype (A1A1) in the maternal blood was higher in PTD cases (34%) as compared to the control subjects (28%). When the wild genotype A1A1 (OR = 1.00) was taken as the reference category, the frequency of A1A2 (OR = 0.815 95% CI = 0.429-1.54, p = 0.531), A2A2 (OR = 0.551 95% CI = 0.193-1.572, p = 0.265) and A1A2/A2A2 (OR = 0.769 95% CI = 0.41-1.43, p = 0.465) was not found significantly associated with PTD (**Table 2**).

Significantly high levels of α-HCH (p = 0.003), β-HCH (p = 0.014), and γ-HCH (p = 0.001) were found in the maternal blood of the PTD cases as compared to the controls. Although higher levels of the other pesticides were found in the maternal blood in the PTD cases than the controls, still the difference in their level was not significant (**Table 3**). The effect of gene environmental interaction on the period of gestation (POG) was studied. Significant interaction was obtained between β and γ-HCH and *CYP17* genotype A1A2 and reduction in POG. When the *CYP17* gene was heterozygous type (A1A2), increasing level of γ-HCH (> 50th percentile) in the maternal blood resulted in an estimated reduction in POG. The interaction term of *CYP17*

Table 2. Genotypic distribution of *CYP17* gene in the maternal blood of Control and PTD Cases

Genotype	Control (n = 100)	PTD (n = 100)	Odds Ratio	95% CI	*p* value
A1A1	28 (28%)	34 (34%)	Ref.		
A1A2	20 (20%)	25 (25%)	0.815	0.429-1.54	0.531
A2A2	6 (6%)	12 (12%)	0.551	0.193-1.572	0.265
A1A1/A2A2	72 (72%)	66 (66%)	0.769	0.41-1.43	0.465

Univariable logistic regression analysis was used to determine the OR and 95% CI, groups have been taken as dependent and polymorphic variants as independent covariates.

Table 3. Levels of OCPs (ppb) in maternal and cord blood of control and PTD subjects

OCP levels (ppb)	Control (n = 100) Mean ± SD	PTD (n = 100) Mean ± SD	*p* value
Maternal Blood			
α-HCH	3.11±3.20	5.32±3.98	0.003*
β-HCH	2.96±4.09	6.65±6.58	0.014*
γ-HCH	4.10±3.56	9.92±6.22	0.001*
p,p'-DDT	2.05±2.28	2.55±2.30	0.096
p,p'-DDE	0.71±0.38	0.81±0.62	0.824
Endosulfan	2.28±1.90	2.84±1.96	0.313
p,p'-DDD	ND	0.30±0.69	ND

*P < 0.05 was considered as significant. Unpaired t test was applied to compare the level of OCPs levels in maternal blood of PTD cases and FTD controls.

and γ-HCH levels has been calculated as follows, assuming adjusted co-variants are constant: POG = B0 + B1 × γ-HCH + B2 × *CYP17* + B3 × *CYP17* × γ-HCH, assuming γ-HCH ≥ 50th percentile = 1, γ-HCH < 50th percentile = 0, *CYP17* A1A1 = 0, *CYP17* A1A2/A2A2 = 1. From **Table 4**, B1 = 1.12, B2 = -0.079, B3 = -2.40. When γ-HCH < 50th percentile and *CYP17* A1A1 = Ref. (B0) = mean POG. When γ-HCH ≥ 50th percentile and *CYP17* A1A1 = B0 + B1 × γ-HCH = B0 + (1.12) = 1.12 (reduction in POG). When γ-HCH < 50th percentile and *CYP17* A1A2/A2A2 = B0 + (-0.079) = -0.079 (reduction in POG). When γ-HCH ≥ 50th percentile and *CYP17* A1A2/A2A2 = B0 + (1.12-0.079-0.2.40) = -1.35 (approx 9 days reduction POG).

Discussion

The role of genetic susceptibility and gene-environment interactions in preterm delivery has largely been unexplored. Growing evidence indicates that familial or intergenerational factors influence preterm delivery. This influence may reflect shared environmental factors or genetic factors, or both. With recent advances in human genetics and molecular biology, assessment of genetic contributions to human diseases has progressed significantly, but the number of studies in this area is limited.

In the present study we have found significantly ($p < 0.05$) higher levels of α-HCH, β-HCH, and γ-HCH in the maternal of the PTD subjects when compared to controls. This is in line with the earlier reports including those from our laboratory where high levels of OCPs were found in PTD as compared to the controls [2, 5, 12]. OCPs have been known to alter the normal estrogen-progesterone balance which is necessary for normal pregnancy [13-15]. OCPs have been reported to not only antagonize the effect of the endogenous hormone but also disrupt their synthesis and receptors [16]. On long term exposure, β-HCH has been shown to decrease the level of estrogen receptor α levels [17]. γ-HCH which has been used to treat lice and scabies in the form of shampoos and lotions, is known to suppress follicle stimulating hormone and decrease progesterone synthesis. Progesterone is essential for trophic support to the placental and maintenance of pregnancy.

In the present study we have found that the frequency of wild type of *CYP17* gene (A1A1) was higher in PTD cases (34%) as compared with the controls (28%) but the significant difference of genotypic distribution was not found in the study groups. Recently we have also reported that null genotypes of *GSTM1/GSTT1* are associated with significant increased risk of PTD [18]. The 5'-untranslated promoter region of *CYP17* gene contains a single base pair T-to-C polymorphism that may create a new Sp-1 site (CCACC box) at 34 bp upstream from the initiation of translation and 27 bp downstream from the transcription start site. This polymorphism introduces a restriction site giving rise to two

Table 4. Regression model testing and interactive effect of OCPs (ppb) in maternal blood and CYP17 genotype on the outcome as preterm birth in cases

	Maternal Blood	
	B[b]	*p* value
α-HCH (≥ 50th vs. < 50th)	-0.546	0.640
CYP17 (Heterogygous vs. other)	0.381	0.695
Interaction term[a]	-1.207	0.231
β-HCH (≥ 50th vs. < 50th)	0.351	0.770
CYP17 (Heterogygous vs. other)	0.086	0.919
Interaction term[a]	-2.11	0.019
γ-HCH (≥ 50th vs. < 50th)	1.123	0.298
CYP17 (Heterogygous vs. other)	-0.079	0.922
Interaction term[a]	-2.40	0.007*
p,p'-DDT (≥ 50th vs. < 50th)	-0.860	0.337
CYP17 (Heterogygous vs. other)	0.528	0.506
Interaction term[a]	-0.278	0.797
p,p'-DDE (≥ 50th vs. < 50th)	0.146	0.894
CYP17 (Heterogygous vs. other)	0.369	0.642
Interaction term[a]	-1.031	0.251
Endosulfan (≥ 50th vs. < 50th)	-0.494	0.658
CYP17 (Heterogygous vs. other)	0.556	0.426
Interaction term[a]	-0.198	0.833
p,p'-DDD (≥ 50th vs. < 50th)	-0.396	0.612
CYP17 (Heterogygous vs. other)	0.013	0.982
Interaction term[a]	-0.077	0.904

[a]After adjustment of factors like age, period of gestation, diet, socioeconomic status, source of water supply, living style, parity. [b]B is un-standardized regression coefficient and this represents the mean period of gestation. *p < 0.005 is considered as significant (after Bonferroni correction).

alleles A1 and A2 [19]. CYP17 suppose to regulate the cortisol biosynthesis and potentially induce preterm delivery.

The genetic susceptibility and its interaction with the environmental exposure need to be considered for better understanding of a disease. In our study we found a significant interaction between γ-HCH and *CYP17* A1A2 genotype and PTD. Our findings are in the support of previously reported study by Mustafa et al, 2013 that when *GSTM1* genotype was absent, increasing levels of β-HCH resulted null deletion increases the risk of preterm delivery [2]. Recently we have also reported that β-HCH may interact with xenobiotic metabolizing genes in the etiology of FGR [20]. In our study, though the genotyping distribution of CYP17 were not significantly associated with PTD subjects as compared to the controls, still its interaction with *CYP17* genotype resulted in significant reduction in period of gestation. This observation has been supported by earlier report by Delpisheh et al., 2009 [21]. That although no significant difference in genotype frequencies of *CYP17* gene was observed between the FGR cases and the control subjects, still there was a significant interaction for FGR between the *CYP17* A1A1 genotype and prenatal alcohol consumption (adjusted OR, 1.4, 95% CI; 1.1-1.9, p = 0.04).

This study shows that OCPs may be an important factor in the etiology of idiopathic PTD. The polymorphism in the *CYP17* gene which is involved in the key steps of estrogen synthesis may provide a protective role in the pathogenesis of PTD. High levels of γ-HCH may cause reduction in the period of gestation *CYP17* A1A2 genotype. To the best of our knowledge, this is the first study to investigate the synergistic effect of OCPs and steroid synthesizing *CYP17* gene in the pathophysiology of PTD. Further studies need to be conducted exploring the effect of other environmental pollutants, metabolic/steroid synthesis gene on the risk of PTD.

Acknowledgements

Authors are thankful to Indian Council of Medical Research for providing infra-structure vide ref. no. 5/7/516/11-RHN.

Disclosure of conflict of interest

None.

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