Reproductive Toxicology 57 (2015) 111–120

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

# Inflammatory-mediated pathway in association with organochlorine pesticides levels in the etiology of idiopathic preterm birth

Md. Mustafa[a,1], Neha Garg[b,1], B.D. Banerjee[a,*], Tusha Sharma[a], Vipin Tyagi[a], Sajad Ahmad Dar[c], Kiran Guleria[b], Rafat S. Ahmad[a], Neelam vaid[b], A.K. Tripathi[a]

[a] Environmental Biochemistry and Molecular Biology Laboratory, Department of Biochemistry, Dilshad Garden, Delhi 110 095, India
[b] Department of Obstetrics and Gynaecology, Dilshad Garden, Delhi 110 095, India
[c] Department of Microbiology, University College of Medical Sciences & G.T.B. Hospital (University of Delhi), Dilshad Garden, Delhi 110 095, India

ARTICLE INFO

*Article history:*
Received 13 March 2014
Received in revised form 18 May 2015
Accepted 28 May 2015
Available online 6 June 2015

*Keywords:*
Preterm birth
Women
Organochlorine pesticides
Cytokines
Prostaglandins
Gene–environment interactions

ABSTRACT

Elevated inflammation is a known risk factor in the pathogenesis of PTB. Despite intensive research, the etiology of idiopathic PTB is still unknown. The present study was designed to explore associations of blood concentrations of organochlorine pesticides (OCPs) with inflammatory/antioxidant gene expression, and cytokines and prostaglandin levels in PTB cases. Significantly high levels of α, β-hexachlorocyclohexane (α, β-HCH), dichlorodiphenyldichloroethane (*o'p'*-DDD), dichlorodiphenyldichloroethylene (*p'p'*-DDE), increased expression of cyclooxygenase-2 (COX-2), and decreased expression of manganese superoxide dismutase (Mn-SOD) and catalase (CAT) genes were seen in PTB cases. Also, increased protein levels of interleukin-6 (IL-6) and decreased protein levels of interleukin-4 (IL-4) and prostaglandin F2α (PGF2α) were found in maternal blood of PTB cases as compared to term controls. Elevated levels of β-HCH along with high expression of COX-2 gene or low expression of Mn-SOD or CAT genes were associated with the decrease in the period of gestation (POG).


## 1. Introduction

Worldwide, preterm birth (PTB, <37 weeks of gestation) accounts for more than 60% of perinatal morbidity and mortality and is the leading cause of neonatal deaths [1]. Globally, every year, around 13 million babies are born preterm, with rates being highest in low and middle-income countries [2]. In a 2012, WHO report, India was shown to be leading among all nation**s** surveyed for number of PTB [3], with incidence of preterm labor around 20% and that of PTB, 10–69% [4]. Although, tocolytic therapy for prevention of PTB has been used for more than four decades, the rate of PTB has not changed, in fact, is increasing [5]. It continues to be a major clinical and public health problem and is regarded as a syndrome with multiple causes. Factors like infections, cervical insufficiency, hormonal imbalance and genetic predisposition are known risk factors for PTB. Association of other risk factors like oxidative stress, environmental factors and gene–environment interaction with PTB is under intense research.

Pesticides are widely used chemicals in agriculture and public health program in India. Among the various pesticides used in India, 40% of them belong to organochlroine class [6,7]. Earlier studies, including those from our laboratory, have reported that blood OCP concentrations are directly associated with the risk of PTB [8–10]. In the previous studies, OCP residues have been **measured** in human blood, amniotic fluid, semen, breast milk and placenta [9–12]. These pesticides accumulate in lipid-rich tissue because of their strong lipophilic nature and slow biodegradability [10]. Daglioglu et al. have reported the concentration of OCPs in female adipose tissue was significantly higher than in males, and OCPs may accumulate more in women due to a higher percentage of body fat [13]. Women may also have metabolic differences that account for differential clearance or longer half life ($t_{1/2}$). Thus women have the potential to have greater risks from exposure to OCPs. β-HCH, endosulfan, dichlorodiphenyltrichloroethane (DDT) and its metabolites may act as endocrine disrupting chemicals (EDCs) and may affect epigenetic and also oxidative stress due to high free radical generation. India is one of the largest producer and consumer of endosulfan in the world. It has been found to be associated with delays in sexual maturity and interfere with sex hormone synthesis among boys

*Abbreviations:* PTB, preterm birth; POG, period of gestation; DDT, dichlorodiphenyltrichloroethylene; DDD, dichlorodiphenyldichloroethane; DDE, dichlorodiphenyldichloroethylene; HCH, hexachlorocyclohexane; IL, interleukins; OCPs, organochlorine pesticides; PGF2α, prostaglandin F2 alpha; COX-2, cycloxiginase-2; CAT, catalase; Mn-SOD, manganese superoxide dismutase.

* Corresponding author. Tel.: +91 11 22135362; fax: +91 11 22590495.
*E-mail address:* banerjeebd@hotmail.com (B.D. Banerjee).
[1] Contributed equally in the manuscript.

http://dx.doi.org/10.1016/j.reprotox.2015.05.018
0890-6238/

in Kasargod District, Kerala, India [14]. β-HCH with the mixture of other OCPs in adipose tissue adjacent to breast carcinoma generate an estrogenic microenvironment through estrogen receptor (ER-α) activation [15] and may disturb the normal estrogen-progesterone balance which is important in the maintenance of pregnancy.

Cyclooxygenase-2(COX-2) enzyme, acts as a link between inflammation and PTB through its involvement in the synthesis of prostaglandins like PGE2 and PGF2α [16]. Increased expression of COX-2 gene and subsequent elevated production of prostaglandins in fetal membranes precedes the onset of normal labor and is associated with PTB [17]. The environmental pollutants like *o,p'*-DDT dose-dependently increases the level of COX-2 mRNA expression and thereby increases the production of prostaglandins in murine RAW 264.7 macrophages has been reported earlier [18]. Increased inflammation occurs in normal parturition, and inflammatory cytokines are comparatively higher in women who deliver preterm [19]. The major sources of inflammatory cytokines are leukocytes, glandular epithelial cells and surface epithelial cells, T-Cells, etc. [20]. Mn-SOD and CAT are antioxidant enzymes that protect cells against reactive oxygen species (ROS) and exaggerated inflammation, which helps in maintaining the physiological redox status of the body [21]. Deficiencies of these antioxidant enzymes were previously associated with PTB cases [16,22].

This study was aimed at (i) estimation of OCPs, pro-inflammatory (IL-2 and IL-6), anti-inflammatory (IL-4 and IL-10) cytokines and PGF2α at protein levels (ii) expression of COX-2, Mn-SOD, and CAT genes at mRNA level (iii) evaluate the correlations of blood OCP concentrations with the mRNA expression, cytokines and prostaglandin levels and risk assessment of PTB.

## 2. Materials and methods

### 2.1. Study subjects

In the present case-control study, we included fifty ($n = 50$) women in spontaneous labor and delivering live births at <37 weeks of gestation (preterm) as cases and fifty ($n = 50$) women in spontaneous labor delivering live births at >37 weeks of gestation (term) as controls. The study was conducted at University College of Medical Sciences (UCMS) associated with Guru Teg Bahadur Hospital (GTB), University of Delhi, Delhi, India during the period of August 2010 to August 2012. Study subjects confirmed their participation by signing an informed consent and the study was approved by the Institutional Ethics Committee for Human Research (IEC-HR), UCMS and GTB hospital, University of Delhi, India vide reference letter dated 27.03.2010.

Women recruited in the study were between 18 and 35 years of age, with BMI between 19 and 26 kg/m$^2$, no history of smoking or occupational exposure to pesticides. The BMI was measured according to the pre-pregnancy weight of the women. All subjects had spontaneous onset of active labor with intact membranes and delivered appropriate for-gestational-age-neonates. Women with a history of one or more spontaneous PTB or late second trimester spontaneous abortion or cervical cerclage were excluded from the study. Women pregnant with multiples, with IUGR, stillborn, other loses, deliveries by scheduled c-section, anemia, diabetes, hypertension, chronic diseases, urinary tract infections, chorioamninitis, recent intake of any anti-inflammatory drugs/steroids or having any complications during pregnancy, and/or labor were excluded from both the groups. For each case, an age, weight and parity matched, low risk pregnancy control delivering an appropriate for gestational age neonate at term was recruited preferably on the same day.

### 2.2. RNA Isolation and synthesis of complementary DNA (cDNA)

In all of the subjects, 4 mL of maternal blood was drawn; 2 mL was collected in EDTA vials and 2 mL in plain vials at the onset of active labor. The blood samples with anticoagulant was fixed with TRI Reagent BD (Sigma-Aldrich, St. Louis, MO, USA), stored at -80$^0$C and were processed within two to three days for total RNA isolation as per the manufacturer protocol (Sigma-Aldrich, St. Louis, MO, USA). The concentration and purity of total RNA was determined using Nano Drop 1000 spectrophotometer (ThermoFisher Scientific Inc, Waltham, MA, USA). RNA quality and integrity were determined via the ratio of absorbance at 260/280 nm, with values between 1.8 and 2.0. Further, quality of isolated total RNA was also checked on agarose gel electrophoresis by mixing with RNA gel loading dye (ThermoFisher Scientific Inc, Waltham, MA, USA). The mixture was kept at 70 °C for 10 min and then immediately placed on ice and loaded on 1.5% agarose gel. The 28s rRNA band was found more intense than the 18s rRNA and there was no any intense smearing down from the 28s and 18s bands. Any sample having degraded RNA was considered as low quality RNA and was excluded from the study. On the same day, total RNA (1 μg) was converted into single strand complementary DNA (cDNA) using Maxima first strand cDNA synthesis kit (ThermoFisher Scientific Inc, Waltham, MA, USA) according to manufacturer's protocol. cDNA was further diluted four times in nuclease free water (ThermoFisher Scientific Inc, Waltham, MA, USA) before use in the Real time quantitative polymerase chain reaction (RT-qPCR). The volume of single strand cDNA did not comprise more than 1/10 of the total PCR reaction volume.

### 2.3. Real-time quantitative polymerase chain reaction experiment

A RT-qPCR experiment was conducted to measure the expression of inflammatory and antioxidant genes. The reactions were performed on Rotor-Gene Q, RT-qPCR with 36-well rotors (Qiagen, 12 Colton Road, East Lyme, USA). Briefly, the PCR amplification master mix of 45 μL contained 6.75 μL of diluted cDNA, 10 μL of PyroStart fast PCR master mix (ThermoFisher Scientific Inc, Waltham, MA, USA), 10 pmol each of forward and reverse specific primer pairs, SYTO®9 Green Fluorescent Nucleic Acid Stain ((ThermoFisher Scientific Inc, Waltham, MA, USA), and 4 μL of nuclease free water. 20 μL of master mix out of 45 μL was dispensed into two PCR tubes. Duplicate of each sample along with no template control wells were kept for PCR amplification. The primer sequence used for COX-2 gene was 5′-TCC TCC TGT GCC TGA TGA TTG C-3′, 5′-ACT GAT GCG TGA AGT GCT GGG-3′. The thermal profile consisted of initial denaturation for 1 min at 95 °C, 40 cycles of 30 s at 95 °C denaturing, 30 s at 50 °C, and 30 s at 72 °C extension. For Mn-SOD and CAT genes the primers sequences were 5-′TGG ACA AGT ACA ATG CTG AG 3′, 5′-TTA CAC GGA TGA ACG CTA AG-3′and 5′-GCC CTG GAA CCT CAC ATC AAC-3′, 5′-CAA CGC CTC CTG GTA CTT CTC-3′, respectively, and annealing temperatures were 55 and 50 °C, respectively. In this analysis, gene expression normalization was done by using GAPDH, a constitutive gene and the primer sequences were 5′-ACC CAC TCC TCC ACC TTT GAC-3′, 5′-TCC ACC ACC CTG TTG CTG TAG-3′. The primer sequences were adopted from Hofmann et al. [23]. These nucleotide sequences were further conformed for its gene specificity by NCBI BAST search program. The above primer sequences were custom synthesized from Sigma-Aldrich, St. Louis, MO, USA. Cumulative fluorescence was measured at the end of extension phase of each cycle. The amplified product was confirmed for specification by melt curve analysis using Rotor-Gene Screen Clust HRM Software (Qiagen, 12 Colton Road, East Lyme, USA). The amplified product was also run on 2% agarose gel (Hi-Media, Paris, France) to check the specific amplification. Relative quantification