Expression of prostaglandin receptors in *Chlamydia trachomatis*-infected recurrent spontaneous aborters

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A study was undertaken to quantify the expression of prostaglandin (PG) receptors and find the effect of gestational age on expression of PG receptor genes in *Chlamydia trachomatis*-infected recurrent spontaneous aborters (RSA). Endometrial curettage tissue (ECT) was collected from 130 RSA (Group I) and 100 age-matched controls (Group II) at the Department of Obstetrics and Gynecology, Safdarjung Hospital, New Delhi (India). PCR was performed for diagnosis of *C. trachomatis* cryptic plasmid; mRNA expression of PG receptor genes was assessed by real-time PCR (q-PCR), while serum progesterone/estrogen levels were determined by respective commercial kits. Data were evaluated statistically. A total of 15.4 % RSA (Group I) were diagnosed as *C. trachomatis*-positive (200 bp), whereas controls were uninfected. q-PCR showed significant upregulation (*P*<0.0001) of PGE2 (EP-1, EP-2, EP-3, EP-4), PGF2α (FP) and PGI2 (IP) receptors in Group I versus Group II. The expression of PG receptors increased significantly with advanced gestational age (*P*<0.002); however, only contractile receptors, EP-1, EP-3 and FP, were positively correlated with gestational age in Group-I. In infected RSA, mean serum progesterone level was significantly low (*P*<0.0001) while serum oestrogen was high (*P*<0.0001). Overall, the data suggest that increased expression of PG receptors, particularly contractile gene receptors (EP-1, EP-3, FP), with advanced gestational age and altered steroid levels could be a possible risk factor for abortion in *Chlamydia*-infected RSA.

INTRODUCTION

Recurrent spontaneous abortion is a frequent outcome in pregnancy and is one of the most difficult areas in reproductive medicine, since the etiology is often unknown. Currently, the role of infectious agents in recurrent loss is not clear and is under active investigation. Genitourinary tract or systemic infection with Gram-negative bacteria in pregnant women can cause abortion and several other perinatal complications (Penta *et al*., 2003; Lamont & Sawant, 2005). Despite a plethora of literature describing an association between *Chlamydia trachomatis* infection and spontaneous abortion (Rastogi *et al*., 2000; Wilkowska-Trojniel *et al*., 2009; Jahromi *et al*., 2010), there is a paucity of mechanistic studies to date on the underlying immunomolecular pathway leading to recurrent spontaneous abortion in infected women.

Uteroplacental prostaglandins (PGs) are lipid mediators that play a pivotal role in reproduction and maintenance and/or termination of pregnancy (Ni *et al*., 2002). PGs mediate the signs/symptoms of Gram-negative septic shock, stimulate myometrium and are capable abortifacients (Silver *et al*., 1995; Hertelendy & Zakar, 2004). Lipopolysaccharide (LPS)-induced abortion has also been found associated with PGs (Skarnes & Harper, 1972). The latter exert their effect through G protein-coupled receptors, designated EP, FP, DP and IP (Narumiya *et al*., 1999). When these receptors are bound by their appropriate prostanoid ligand, they activate contractile (EP-1, EP-3, FP)/relaxatory (EP-2, EP-4, IP) receptors. It has been suggested that...
changes in the expression of PG receptors throughout gestation and with labour may participate in maintenance of uterine quiescence for the majority of gestation and the switch to contractions at delivery for expulsion of the fetus (Coleman et al., 1994). Previous studies of PG receptor gene expression are limited to non-primate species or lower segment biopsies from pregnant women.

Furthermore, endocrine factors like progesterone and oestrogen have also been implicated in the etiology of recurrent abortion, with poorly understood roles. In pregnancy, progesterone is in dynamic balance with oestrogen in the control of uterine activity. A fetal endocrine cascade involving an increase in oestrogen and decrease in progesterone in maternal plasma triggers the onset of labour (Challis et al., 2000). This endocrine cascade ultimately leads to both the activation and stimulation of the myometrium through the increased production of stimulatory PGs. Thus, low progesterone values are associated with miscarriage and ectopic pregnancy, both considered as non-viable pregnancies. Also, there is increasing evidence that progesterone exhibits anti-inflammatory activities (Piccinni et al., 2001).

In an earlier study, we found an upregulation of cox-2 and increased PGE2 synthesis in C. trachomatis-positive recurrent spontaneous aborters (RSA) (Singh et al., 2015). However, differences in mRNA expression of contractile/relaxatory PG receptors were not studied. As there are multiple sub-types of PG receptors mediating opposite effects, we hypothesized that variations in the expression of PG receptors, namely EP-1, EP-2, EP-3, EP-4, FP and IP, may have an important role leading to abortion in Chlamydia-infected women. The present study aimed to quantify the mRNA expression of PG contractile/relaxatory receptor genes in RSA and sought to find the effect of gestational age on the expression of PG receptors in C. trachomatis-infected women.

**Fig. 1.** Increased expression of various PG receptors. (a) EP-1, EP-3, (b) EP-2, EP-4, (c) FP and (d) IP in C. trachomatis-positive RSA versus controls by q-PCR. Comparisons between the two groups were made with the non-parametric Mann–Whitney test; ***P<0.0001. CT+ve, C. trachomatis-positive.

**METHODS**

**Study population and enrolment of patients.** A prospective study was performed in 130 women experiencing recurrent spontaneous abortion in their first trimester of pregnancy (Group I) while 100 age-matched healthy asymptomatic pregnant women undergoing induced abortion for medical termination of pregnancy served as controls (Group II). All patients in Group I were symptomatic and presented with bleeding per vagina, abdominal pain, passage of clots, etc., and underwent dilatation and evacuation for incomplete spontaneous abortion at the Department of Obstetrics and Gynaecology, Safdarjung Hospital, New Delhi, India. The study had hospital ethical committee permission and prior informed written consent was taken from each patient. Those with local causes of bleeding/prior history of genitourinary infection/recent antibiotic therapy/HIV-positivity/VDRL-positivity/history of recently treated sexually transmitted infection other than C. trachomatis/genital tuberculosis were excluded. Information on obstetric/medical/gynaecological history including last menstrual period, gravidity/parity-abortion history and past genital/urinary tract infection was collected in standardized questionnaires from all patients enrolled.

**Collection and processing of clinical samples.** Endometrial curetage tissue (ECT) samples were collected from patients of Groups I and II and transported in PBS on ice to the Microbiology laboratory at the National Institute of Pathology (ICMR), New Delhi, India, for scientific investigations. One part of the tissue was fixed in neutral buffered formalin and paraffin-embedded for histopathological confirmation of the tissue; another part of the ECT was utilized for DNA extraction while the remaining tissue was stored in RNA later (Sigma Aldrich) at −80 °C for quantitative analysis of mRNA expression. Non-heparinized blood (3–5 ml) was also collected from each patient for separation of serum. The latter was stored at −20 °C until assayed.

**DNA isolation.** DNA was isolated from the ECT utilizing a commercial kit (Wizard Genomic DNA Isolation kit, Promega) as per the manufacturer’s recommendations. Briefly, the tissue was homogenized in liquid nitrogen, treated with lysis buffer and the lysate was, thereafter, treated with protein precipitation solution. Finally, the DNA was precipitated by 2-propanol.

**PCR detection of C. trachomatis.** PCR assay was performed for the diagnosis of C. trachomatis infection in both groups. For this, amplification reactions using a DNA Thermal Cycler (Applied Biosystems) were set up in 25 µl reaction volumes in 0.2 ml PCR tubes. The reaction mix consisted of 1 µg genomic DNA, 0.2 mM of each dNTP (Fermentas), 10 PM of each oligonucleotide (Fermentas), 2.5 µl 10× PCR buffer (Fermentas) and 1 unit of Taq DNA polymerase (Fermentas). The genomic DNA was initially denatured at 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s, primer annealing for 1 min at 52.4 °C for beta-globin (internal control) or at 57.3 °C for C. trachomatis cryptic plasmid, and extension at 72 °C for 45 s. Final extension was carried out at 72 °C for 7 min to obtain a product of 268 bp or 200 bp for beta-globin and C. trachomatis cryptic plasmid, respectively (George et al., 2003). The amplified product was confirmed by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized under UV transillumination.

**Quantitation of PG receptors.** Quantitative real-time PCR (q-PCR) assay was performed on a 7000 Real-time PCR system (Applied Biosystems) using ECT for studying the quantitative expression of various PG receptor genes, viz. PGE2 (EP-1, EP-2, EP-3, EP-4), PGF2α (FP) and
PGI2 (IP). cDNA (1.5 µg µl⁻¹) was used for q-PCR. Briefly 10 µl Taq-man master mix, 1 µl probe, 4 µl cDNA and 5 µl nuclease-free water were mixed to make up a final volume of 20 µl. The reactions were set in duplicates. The assay was standardized for the internal control gene β-actin in the ECT and those samples showing consistency with β-actin were selected for real-time PCR assay. The threshold cycle (Ct) value was calculated as mean Ct target genes for each sample by using SDS software and the relative quantification was used to measure gene expression by relating the PCR signal.

**Estimation of serum progesterone.** Serum progesterone concentration was determined by a commercial progesterone EIA kit (Cayman) as per the manufacturer’s guidelines. Briefly, 100 µl EIA buffer was added to the non-specific binding wells followed by the addition of 50 µl EIA buffer and 50 µl of standard dilutions to the dilution wells. After the subsequent addition of 50 µl samples per well, each sample was assayed at a minimum of two dilutions and each dilution was assayed in duplicate. Thereafter, 50 µl of progesterone AChE tracer, followed by 50 µl of antiserum was added to each well followed by incubation, washing, addition of Ellman’s reagent and incubation at room temperature. Finally the plate was read at wavelengths between 405 and 420 nm.

**Estimation of serum oestrogen.** Serum oestrogen concentration was determined by a commercial oestradiol ELISA kit (Cayman) as per the manufacturer’s guidelines. Briefly, 100 µl ELISA buffer was added to the non-specific binding wells followed by the addition of 50 µl ELISA buffer and 50 µl of standard dilutions to the dilution wells. After the subsequent addition of 50 µl samples per well, each sample was assayed at a minimum of two dilutions and each dilution was assayed in duplicate. Thereafter, 50 µl of oestradiol AChE tracer, followed by 50 µl of oestradiol ELISA antiserum was added to each well except the non-specific binding and blank wells followed by washing, addition of 200 µl Ellman’s reagent and incubation at room temperature. Finally the plate was read at wavelengths between 405 and 420 nm.

**Statistical analysis.** Statistical analysis was performed using the GraphPad prism software (version 5.0). Fisher’s exact test, Mann–Whitney test, one-way ANOVA and Spearman’s rank correlation coefficient were used to calculate the level of significance within the groups.

**RESULTS**

**Diagnosis of C. trachomatis in ECT**

One hundred and thirty RSA with two or more abortions constituted the study group (Group I). The average gestational age of women (Groups I and II) was 8 weeks (first trimester). Overall, 15.4% (n=20) women experiencing recurrent spontaneous abortion were diagnosed as C. trachomatis-positive for the cryptic plasmid by PCR assay in Group I. However, none of the control patients in Group II were found to be Chlamydia-infected (Fisher’s Exact test; P<0.0001).

**Expression of PG contractile/relaxatory receptors**

In order to investigate whether chlamydial infection affects the functionality of the PG contractile/relaxatory receptors, the expression of PGE2 (EP-1, EP-2, EP-3, EP-4), PGF2α

Fig. 3. Correlation between gestational age and EP-1, EP-2, EP-3, EP-4 and FP receptors in C. trachomatis-positive RSA. Results were analysed by Spearman rank correlation coefficient (r=0.903, 0.96 and 1.00, respectively).
(FP) and PGI2 (IP) receptor genes was studied in the ECT at the transcript level by Taqman q-PCR and the expression was compared between patient groups as a ratio to the expression of the constitutively expressed housekeeping β-actin gene in C. trachomatis-positive RSA. Analysis of C, values showed that the mRNA expression of PGE2 contractile receptors EP-1/EP-3 and relaxant receptors EP-2/EP-4 was significantly increased in comparison with controls (non-parametric Mann-Whitney test; P<0.0001) (Fig. 1a). Analysis of the gene expression of the PGF2α contractile receptor FP showed that the gene expression was significantly higher in C. trachomatis-positive RSA versus controls (non-parametric Mann–Whitney test; P<0.0001) (Fig. 1d). The PGI2 relaxant receptor gene, IP, was also found to be increased significantly in the infected patients in comparison with controls (non-parametric Mann–Whitney test; P<0.0001) (Fig. 1d).

A significant effect of gestational age was observed on the mRNA expression of various PGE2, viz. EP-1, EP-2, EP-3, EP-4, PGF2α (FP) and PGI2 (IP), receptor genes in the infected women (one-way ANOVA non-parametric Kruskal–Wallis test; P<0.002) (Fig. 2a–f). The PG receptor genes (EP-1, EP-2, EP-3, EP-4, FP and IP) were further correlated with gestational age. The r value was found to be significant for all the receptors. The contractile EP-1 and EP-3 receptors were positively correlated (r=0.903; P=0.0028 and r=0.96; P=0.0021, respectively) (Fig. 3a, c) while the relaxant EP-2 and EP-4 receptors were found to be negatively correlated with gestational age (r=-0.94; P=0.017 and r=-0.98; P=0.0008, respectively) (Fig. 3b, d) in infected RSA. Similar correlation was also made between the FP receptor and gestational age in C. trachomatis-infected spontaneous aborters (Group I). The FP gene was found to be significantly positively correlated with gestational age (r=1.00; P=0.002) (Fig. 3e); however, the IP receptor gene showed no correlation.

**Concentration of serum progesterone and oestrogen in recurrent aborters**

Mean serum progesterone level was estimated in controls and C. trachomatis-positive as well as uninfected RSA and it was found that the progesterone concentration was significantly low (20.2 ng ml⁻¹) in the C. trachomatis-positive RSA, as compared with both uninfected RSA (40 ng ml⁻¹, Mann–Whitney test, P<0.002) and the control group (145.48 ng ml⁻¹; Mann–Whitney test, P<0.002) (Fig. 4a).

Mean serum oestrogen concentration was significantly high (458.26 pg ml⁻¹) in the C. trachomatis-positive RSA, as compared with both uninfected RSA (218.58 pg ml⁻¹, Mann–Whitney test, P<0.0001) and the control group (65.56 pg ml⁻¹; Mann–Whitney test, P<0.0001) (Fig. 4b).

**DISCUSSION**

Sexually transmitted C. trachomatis genitourinary infection is a widespread public health concern globally including in India because of its increased prevalence (Rastogi et al., 2003, 2014; Mittal et al., 2004; Reddy et al., 2004) and potentially devastating reproductive consequences in females, particularly in relation to adverse obstetric outcomes such as RSA. Although the immunopathologic consequences of infection and the adverse effects that C. trachomatis has on the female genital tract are well established, the underlying mechanism of Chlamydia-induced spontaneous abortion in women warrants further research.

The PGE2 receptors (EP-1, EP-2, EP-3 and EP-4) are present in the human myometrium tissue during pregnancy
and at term, levels of both systemic and local PGE2 increase dramatically (Leonhardt et al., 2003). A recent study has suggested that chlamydial infection leads to an upregulation of *cox-2* in infected RSA which probably mediates an increased PG synthesis (Singh et al., 2015). Animal studies have reported an association between *C. psittaci* infection and the intrauterine release of PGE2 in sheep leading to preterm labour (Howie et al., 1989). It has been further suggested that changes in the expression of PG receptors could be involved in the maintenance of uterine quiescence for the majority of gestation and, possibly, may activate the uterus to contract at the time of parturition for expulsion of the fetus (Brodt-Eppley & Myatt, 1998; Smith et al., 2001). Studies have shown that two- to three-fold increase in uterine and ovarian PG concentrations coincided with the induction of *cox-2* (Gross et al., 2000). Also, PGE2 is generated in abundance at the sites of infection and inflammation as a result of the rapid upregulation of *cox-2* (Koeberle & Werz, 2009). PGF2α has also been considered as the primary candidate present during pregnancy, where it plays a crucial role in the myometrium during parturition by increasing the oxytocin-induced contractions. Furthermore, an increase in intrauterine PGF2α concentration in pregnant mice treated with LPS was demonstrated and it was concluded that systemic administration of PGE2 and PGF2α resulted in murine fetal death (Skarnes et al., 1972). In contrast, little is known of the expression pattern and function of the IP receptor in the human endometrium, although prostacyclin synthase and IP receptor expression have been demonstrated in pregnant and non-pregnant myometrium (Giannoulias et al., 2002). However, to the best of our knowledge, none of the studies to date has elucidated the endometrial expression of PG contractile/relaxatory gene receptors in the ECT of women having history of recurrent spontaneous abortion and found to be harbouring *C. trachomatis* infection. This study demonstrated for the first time significantly altered expression of contractile/relaxatory PG receptor genes in the ECT of *C. trachomatis*-positive RSA, thereby confirming the involvement of PG synthesis in abortion.

Also, a significant positive correlation was observed between advanced gestational age and contractile receptors, viz. EP-1, EP-3 and FP, as evident by their increased mRNA expression. In humans, EP receptor mRNA expression may be temporally expressed with respect to gestation (Leonhardt et al., 2003; Brodt-Eppley & Myatt, 1999; Astle et al., 2005), PGE2 dampens maternal immune responses against fetal tissues (Kvirkvelia et al., 2002). A few studies have also demonstrated a tendency towards decreased EP-2 receptor mRNA expression with advancing gestational age in humans (Brodt-Eppley & Myatt, 1999) and also in rat myometrium (Leonhardt et al., 2003), while an increased EP-1 mRNA expression with advanced gestational age in baboon cervix was reported (Smith et al., 2001). In our study, we found EP-1, EP-3 and FP receptors to be positively correlated with gestational age in *C. trachomatis*-positive RSA; however, the expression of EP-2 and EP-4 was negatively correlated. Further research should focus on the control of expression of the EP-1, EP-3 and FP receptor genes in such women.

A well-developed placenta secretes adequate amounts of oestrogen and progesterone. Both these hormones are responsible for maintaining the ovum during its early growth period. If their secretion is inadequate, early abortion may result. The endocrinology of infectious abortion has been the subject of intermittent attention; however, this is restricted largely to animal studies. Studies in mice (Saksena & Lau, 1973) and in guinea pigs (Blatchley et al., 1972) show that progesterone as well as oestrogen are capable of causing synthesis and/or release of PGs from the uterine tissue. LPS alters the serum level of progesterone and oestrogen during the preimplantation days of pregnancy and elevates the oestrogen/progesterone ratio in the mouse (Agarwal et al., 2010). It was further confirmed by these investigators that an altered oestrogen/progesterone ratio in serum during preimplantation in mouse in response to the bacterial infection transformed the uterine receptivity to a refractory state resulting in unsuccessful pregnancy. Abortion in late gestation during experimental infection of ewes after infection with *C. psittaci* is reportedly due to the secretion of cortisol by the fetal adrenals followed by an elevation of circulating oestrogens and PGs and a drop of progesterone in maternal plasma (Bosc et al., 1981), which was further confirmed by other investigators also (Leaver et al., 1989). *C. trachomatis* infection of the trophoblast showed a relative increase in heat shock protein 60 compared with major outer membrane protein, suggestive of chronicity and inflicted injury resulting in an impaired trophoblast endocrine function (Azenabor et al., 2007). This study also emphasized that *C. trachomatis* plays an aetiologic role in the pathogenesis of disturbed pregnancies and reported compromised oestrogen and progesterone biosynthesis in *C. trachomatis*-infected trophoblast.

The combination of oestrogen and progesterone seems to stimulate PG production in ovariectomized animals (Saksena & Lau, 1973). Changes in the concentrations of oestrogen and progesterone influence the release of PGE2 at parturition (Olson et al., 1984). However, to date, there is no clear consensus between PG production and hormone output in RSA. The available literature has focused largely on progesterone; an *in vitro* study concluded that decline in PG and rise in progesterone occur independently of each other (Fowkes et al., 2001) while others have reported negative regulation of PG receptors (PGE2/PGF) by progesterone (Ishihara et al., 1995). In our study, an increased expression of PG receptors, low progesterone and increased oestrogen levels were found in *C. trachomatis*-infected RSA. Various studies have suggested that decreased endogenous progesterone in the uterus increases uterine PG (Hapangama, 2003; Loose & Stancel, 2006) and its supplementation has been widely used to prevent miscarriage. The altered ratio of progesterone:oestrogen, in favour of the latter, upregulates the synthesis of uterine PG and labour (Challis et al., 2000). Oestrogens promote a series of
myometrial changes including increased production of PGE2 and PGF2α (Fuchs et al., 1993).

Results show that chlamydial infection results in an increase in various PG receptors and decreased progesterone level in women experiencing RSA, ultimately leading to an upregulated PG synthesis within the fetal membranes. Our data further show a positive correlation between several contractile gene receptors (EP-1, EP-3 and FP) and gestational age.

Overall, our data suggest that increased expression of PG receptors, particularly contractile gene receptors, with advanced gestational age could be a possible risk factor for abortion in C. trachomatis-infected RSA. Also, there is currently no information on the transcriptional regulation of PG receptor genes. An elucidation of the factors that control the expression of contractile EP-1, EP-3 and FP gene receptors may shed light on the underlying molecular pathway involved in spontaneous abortion among the C. trachomatis-infected women for an improved management of female reproductive life.

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