JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Determination of chlamydial load in recurrent miscarriage in relation to some female sex hormones: a case-control study

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Abstract

Introduction: *Chlamydia trachomatis* is a frequent cause of adverse pregnancy outcomes including recurrent spontaneous abortion (RSA). However, regulation of infectious load by host immune response is unknown. Female sex hormones are known to affect *C. trachomatis* infection. The aim of this study was to determine correlation of chlamydial infectious load and gestational age with concentration of progesterone/estrogen in RSA.

Methodology: Urine and non-heparinized blood were collected from patients with history of > 3 spontaneous abortions (n = 150, cases) and those with history of > 2 successful deliveries (n = 150, controls) from Department of Obstetrics and Gynecology, Safdarjung hospital, New Delhi, India. *C. trachomatis* positivity was determined by polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) and chlamydial load by real-time PCR. Estrogen and progesterone concentrations were estimated by ELISA and correlated with chlamydial load.

Results: 22/150 case patients were positive for *C. trachomatis*. 2,000–10,000 copies/mL of chlamydial load were detected in infected RSA patients. Progesterone concentration showed significant decrease while estrogen concentration was significantly increased in *C. trachomatis*-positive RSA patients versus controls. Chlamydial load and estrogen concentration were positively correlated while progesterone concentration was negatively correlated with chlamydial load. Gestational age was positively correlated with concentration of estrogen and negatively correlated with concentration of progesterone in infected-RSA women.

Conclusions: Overall findings suggest that interplay between chlamydial copy load, hormonal changes such as increased expression of estrogen and decreased expression of progesterone, and advanced gestational age may be contributing as deciding factors for ensuing RSA during *C. trachomatis*-infection.

Key words: *Chlamydia trachomatis*; recurrent abortion; estrogen; progesterone; chlamydial load.

J Infect Dev Ctries 2023; 17(7):999-1006. doi:10.3855/jidc.17677

(Received 16 November 2022 – Accepted 06 February 2023)

Introduction

Recurrent spontaneous abortion (RSA) is one of the most frequently occurring pregnancy-associated complication affecting 2-5% of the population. Its idiopathic etiology makes clinical management and treatment difficult in many cases. The known causes of RSA include chromosomal abnormalities, metabolic and hormonal issues, autoimmune disease and infection [1]. *C. trachomatis* is an intracellular sexually transmitted pathogen with a biphasic life cycle consisting of the elementary body as the infectious form and reticulate body as the metabolically active non-infectious form [2]. The association of *C. trachomatis* and pregnancy complications including RSA has been reported in serum, endometrial tissue and urine in several studies but the mechanistic approach utilized by the pathogen to induce abortion is still under active investigation [3-6]. *C. trachomatis* targets the columnar epithelial cells of the female genital tract. The mucosal barrier, which is made up of epithelial cells and mucus as well as an innate, humoral and cell-mediated immune response, plays a role in host defense against *C. trachomatis* [7]. In pregnant infected women, *C. trachomatis* colonizes the upper genital tract by invading the choriodecidual space causing chorioamnionitis and subsequently abortion or a non-viable pregnancy. Infections have specifically been

linked to 15% of early miscarriages and 66% of late miscarriages [8-9].

The sex hormones estrogen and progesterone regulate host vulnerability to sexually transmitted infections infecting the mucosal surfaces of the female genital tract, as well as innate and adaptive immune responses. Sexually transmitted disease (STD) pathogens that infect this site include viruses such as human immunodeficiency virus (HIV-1), herpes simplex virus (HSV-2) and human papilloma virus (HPV), and bacteria such as *Neisseria gonorrhoeae* and *C. trachomatis* [10].

The roles of endocrine hormones like progesterone and estrogen have been well indicated in the etiology of recurrent abortions [11]. These hormones vary throughout the menstrual cycle regulating ovulation, endometrium proliferation, egg maturation and maintenance of pregnancy if successful fertilization has occurred. In pregnancy, progesterone and estrogen are in balance with each other and responsible for uterine activity and initiating labour. Low progesterone concentration during the first trimester of pregnancy is indicative of a miscarriage or ectopic pregnancy. Sex hormones are known to influence the composition of the vaginal microbial biome [12]. These hormones play an essential role in proper functioning of the female genital tract, therefore their effects on the progression of genital infections such as *C. trachomatis* in pregnancy related complications poses an interesting research question. There are many factors determining the outcome of *C. trachomatis* and hormone interactions such as hormone concentration, area of genital tract infection, etc. [13].

It has also been reported in a few studies that *C. trachomatis* infection and its progression can be altered by the use of hormonal contraceptives which have synthetic hormones as their composition [14-15]. More than 50% of the studies found positive correlation between risk of chlamydial transmission and use of hormonal contraceptive [14]. There are various reasons of unexplained spontaneous abortion, which include altered balance of hormones during pregnancy. It has also been investigated how other reproductive hormones affect fertility and pregnancy outcomes in women with *C. trachomatis* infection. A study examining the levels of reproductive hormones in *C. trachomatis*-associated infertility in women found increased level of luteinizing hormone in *Chlamydia*-infected women as compared to negative fertile women [16]. It has been demonstrated in mice that lipopolysaccharide (LPS) increases the estrogen to progesterone ratio [17]. The present study, therefore, aimed to determine the correlation of chlamydial load and gestational age with the concentration of progesterone/estrogen in infected recurrent spontaneous aborters.

Table 1. Clinical characteristics of participants.

Characteristics	RSA Group (n = 150) (%)	Control (n = 150) (%)
Age (years)		
< 20	35 (24.13)	32 (21.33)
20-25	32 (22.06)	33 (22.00)
26-30	49 (32.66)	41 (27.33)
31-35	34 (23.44)	32 (21.33)
Mean age	29.69 ± 4.02	27.72 ± 3.34
Gravidity		
1	0	0
2	0	102
> 2	150 (100)	48
Avg. gravidity	3	
Parity		
0-1	0	0
≥ 2	0	150
No. of abortions		
0-1	0	0
1-2	0	0
≥ 3	150	0
Menarche (years)		
12-14	128 (88.27)	130 (89.96)
15-16	22 (14.66)	15 (10.34)

RSA: recurrent spontaneous abortion; Avg: Average

Methodology

Ethical approval, patient enrollment and collection of clinical samples

Before the study, ethical permission (IEC/VMMC/SJH/Project/2021-04/CC-126) was obtained from the Ethical Committee of Vardhman Mahavir Medical College (VMMC) and Safdarjung Hospital (SJH), New Delhi (India) and informed written consent was taken from each subject patient before enrollment and sample collection. Clinical samples which included 10-15 mL of urine and 5.0 mL of non-heparinized blood were collected from each patient in the case group comprising of women with history of RSA (Group I; n = 150) and in the control group comprising of asymptomatic women with history of at least two or more successful deliveries (Group II; n = 150) (Table 1). All patients were enrolled at the Department of Obstetrics and Gynecology at SJH. Patients were age-matched and non-pregnant. Patients with anatomical anomalies/ endocrine disorders like thyroid and diabetes/chromosomal disorders/infection with TORCH pathogens (*Toxoplasma gondii, Rubella, Cytomegalovirus, Herpes simplex* virus)/HIV/venereal disease research laboratory (VDRL) positivity were not included.

Detection of various sexually transmitted pathogens

The sexually transmitted pathogens (*Mycoplasma genitalium*, *Ureaplasma urealyticum*/*parvum*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*) were diagnosed in urine by Fast Track Diagnostics Sexually Transmitted Disease real time qPCR kit (Siemens Healthcare, Erlangen, Germany) as per the manufacturer's recommendations provided in the kit.

DNA isolation and detection of Chlamydia trachomatis DNA

The urine pellet was treated with lysis buffer for isolation of DNA. Lysate precipitation was carried out in isopropyl alcohol followed by elution in nuclease-free water [18]. The major outer membrane protein (MOMP) (537 bp) and plasmid (200 bp) genes were targeted to detect the presence of *C. trachomatis* DNA [19] by polymerase chain reaction (PCR) assays. Commercially synthesized primers (GCC Biotech, New Delhi, India) of MOMP [20] and plasmid [21] genes were utilized (Table 2). *C. trachomatis* DNA (Vircell, Granada, Spain) was used as positive control. The amplifications were performed as described earlier [19].

Estimation of Chlamydia trachomatis IgG antibody

Anti-chlamydial IgG antibodies were detected in serum using *C. trachomatis* IgG ELISA kit (Euroimmun, Lubeck, Germany) as per the manufacturer's recommendations. Briefly, 100 µL calibrators, controls and diluted serum samples were transferred to the wells and incubated for 30 minutes at room temperature followed by washing the plate thrice with 300 µL wash buffer to remove any unspecific binding. Thereafter, 100 µL enzyme conjugate (peroxidase antihuman IgG) was added to each well followed by plate incubation for 30 minutes at room temperature. Plates were then washed and 100 µL chromogen solution was added followed by incubation for 15 minutes at room temperature. The reaction was terminated by adding stop solution and the plate was read within 15 minutes at 450 nm in an enzyme-linked immunosorbent assay (ELISA) reader (Titertek, Helsinki, Finland).

Determination of chlamydial load

The chlamydial infectious load was determined by quantitative PCR (Step One plus, Applied Biosystems, Waltham, MA, USA) in *C. trachomatis*-positive urine samples. Serial dilutions of *C. trachomatis* Amplirun DNA was prepared (10000, 1000, 100, 10, 0.1 µL) and a 20 µL reaction was set up consisting of 10 µL of SYBR green master mix, 2 µL of plasmid gene primers (200 bp), 5 µL of *C. trachomatis* diluted DNA (Vircell, Granada, Spain) and 3 µL sterile water. The chlamydial load in each urine sample was determined by plotting the standard curve and the cycle threshold value was computed as the average C_t target gene for each sample.

Estimation of progesterone

Serum progesterone concentration was determined by a commercial progesterone ELISA kit (Calbiotech, El Cajon, CA, USA) as per the guidelines of the manufacturer. 10 µL of progesterone standards, control and patient's serum sample was added into each well followed by 200 µL of progesterone enzyme conjugate. Incubation was carried out for 60 minutes at room temperature followed by washing to prevent non-specific binding. Thereafter, 3,3',5,5'-tetramethylbenzidine (TMB) substrate (100 µL) was transferred to each well and incubated for 15 minutes. Finally, stop solution was pipetted to all wells and the plate was read within 15 minutes at a wavelength of 450 nm in an ELISA reader (Titertek, Helsinki, Finland).

Estimation of estrogen

The concentration of serum estrogen was determined by a commercial estrogen ELISA kit (Calbiotech, El Cajon, CA, USA) as per guidelines of the manufacturer. 10 µL estrogen standards, control and patient's serum sample were added into each well followed by 200 µL of estrogen enzyme conjugate. Incubation was carried out for 60 minutes at room temperature and washing was performed to prevent non-specific binding. TMB substrate (100 µL) was transferred to all wells and incubated for 15 minutes. Finally, stop solution was pipetted to all wells and the plate was read within 15 minutes at a wavelength of 450 nm in an ELISA reader (Titertek, Helsinki, Finland).

Table 2. Primers used for the detection of *Chlamydia trachomatis*.

Gene name	Primer Sequence: 5'-3'	Amplicon size (bp)	Reference
Major outer membrane protein (MOMP)	TAT ACA AAA ATG GCT CTC TGC TTT AT CCC ATT TGG AAT TCT TTA TTC ACA TC	537 bp	Palmer *et al.* (1991) [20]
Plasmid	CTA GGC GTT TGT ACT CCG TCA TCC TCA GGA GTT TAT GCA CT	200 bp	George *et al.* (2003) [21]

Statistical evaluation

The results were analyzed using GraphPad prism software (version 8.0). Mann-Whitney and Spearman's rank correlation tests were utilized to calculate level of significance within the groups. Statistical significance of $p < 0.05$ was accepted.

Results

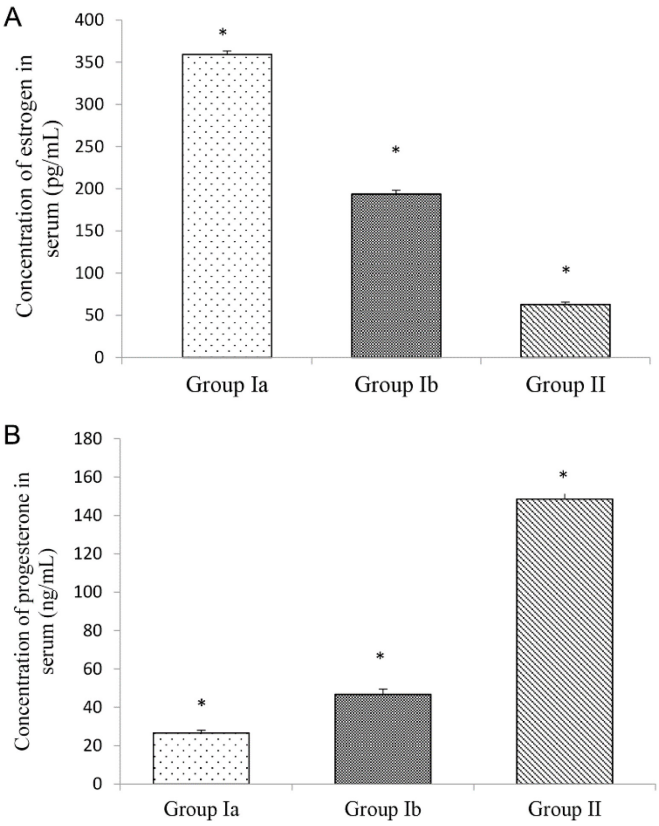
Detection of Chlamydia trachomatis

150 RSA patients with three or more abortions constituted the study group (Group I). Overall, 14.6% (n = 22, Group Ia) women experiencing RSA were diagnosed positive for *C. trachomatis* using cryptic plasmid and MOMP by PCR assays (Figure 1). Patients who were negative for *C. trachomatis* were categorized as Group Ib. However, control patients (Group II) were not found positive for *C. trachomatis*.

Estimation of Chlamydia trachomatis IgG antibody

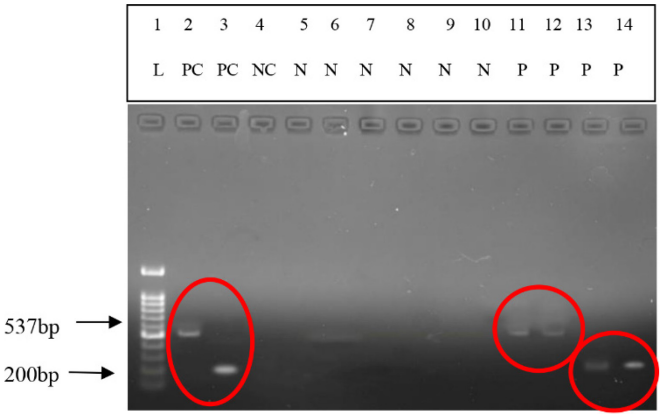
24 patients showed the presence of *C. trachomatis* IgG antibodies (Group I); however, none was positive in control patients (Group II).

Figure 2. A: Serum concentration of estrogen (pg/mL); **B:** progesterone (ng/mL) in *Chlamydia trachomatis*-positive recurrent spontaneous abortion patients and controls.



*p < 0.05; Group Ia: *C. trachomatis*-positive patients; Group Ib: *C. trachomatis*-negative patients; Group II: controls.

Figure 1. Polymerase chain reaction (PCR) for detection of endogenous plasmid (200 bp) and MOMP (537 bp) of *Chlamydia trachomatis* in the urine of recurrent spontaneous aborters.



Lane 1: 100 bp ladder (L), lanes 2 – 3: positive control (PC) for major outer membrane protein (MOMP) and plasmid, respectively, lane 4: negative control (NC), lanes 5 – 10: C. trachomatis-negative DNA samples, lanes 11 – 12: *C. trachomatis*-positive DNA samples for MOMP, lanes 13 – 14: *C. trachomatis*-positive DNA samples for plasmid.

Detection of other sexually transmitted pathogens

2 patients were found to be infected with Cytomegalovirus and *T. gondii*, 1 with HSV1/2 and 2 were infected with Rubella. All these patients were excluded from the study.

Determination of chlamydial load

Quantitative PCR by SYBR green assay was used to determine the chlamydial infectious load in each RSA patient (Group Ia) after evaluating the standard curve drawn by the prepared concentrations of Amplirun. *C. trachomatis* Vircell DNA. 2,000–10,000 copies/mL of *C. trachomatis* was detected in the urine of infected RSA patients.

Detection of serum progesterone and estrogen concentrations in recurrent aborters

Mean serum progesterone level was estimated in *C. trachomatis*-infected RSA and uninfected patients RSA women and controls. Concentration of progesterone was significantly low (26.56 ng/mL) in Group Ia versus Group Ib and Group II (46.73 ng/mL in Group Ib; 148.58 ng/mL in Group II) (Mann–Whitney test $p < 0.05$).

A significantly high concentration of serum estrogen was found (359.14 pg/mL) in Group Ia versus Group Ib and Group II (193.63 pg/mL in Group Ib; 62.82 pg/mL in Group II) (Mann–Whitney test, $p < 0.05$) (Figure 2).

Correlation of Chlamydia trachomatis load with concentrations of serum progesterone and estrogen

Expression of progesterone and estrogen was correlated with the chlamydial load in positive RSA women (Group Ia). Correlation was found to be significantly positive between concentration of estrogen and chlamydial load (r = 0.913; $p < 0.05$; Figure 3a) while it was negatively correlated to concentration of progesterone in Group Ia (r = 0.064; $p < 0.05$; Figure 3b) indicating that greater chlamydial load leads to higher estrogen and lower progesterone expression in infected RSA patients.

Correlation between the concentration of serum progesterone/ estrogen and gestational age

The levels of female sex hormones such as progesterone and estrogen were further correlated with the gestational age of infected RSA patients. Correlation was found to be significantly negative between gestational age and concentration of progesterone in Group Ia (r = 0.676; $p > 0.05$; Figure 4a) while a positive correlation was observed between gestational age and estrogen concentration in Group Ia (r = 0.92; $p < 0.05$; Figure 4b) indicating that concentration of progesterone decreases with increase in gestational age in *C. trachomatis*-infected patients.

Discussion

Reproductive hormones play a critical role in regulation of the menstrual cycle in women. They also precisely coordinate functions of immune activation of the female genital tract. Sex hormones estrogens and progesterone are secreted by the ovary fluctuating in line with menstrual cycle [22]. Menstrual cycle of humans is characterized by the proliferative/follicular phase and the secretory/luteal phase [23]. In the

Figure 3. Correlation between **A:** concentration of progesterone and chlamydial load (copies/mL); **B:** concentration of estrogen and chlamydial load (copies/mL) (Spearman's rank correlation test).

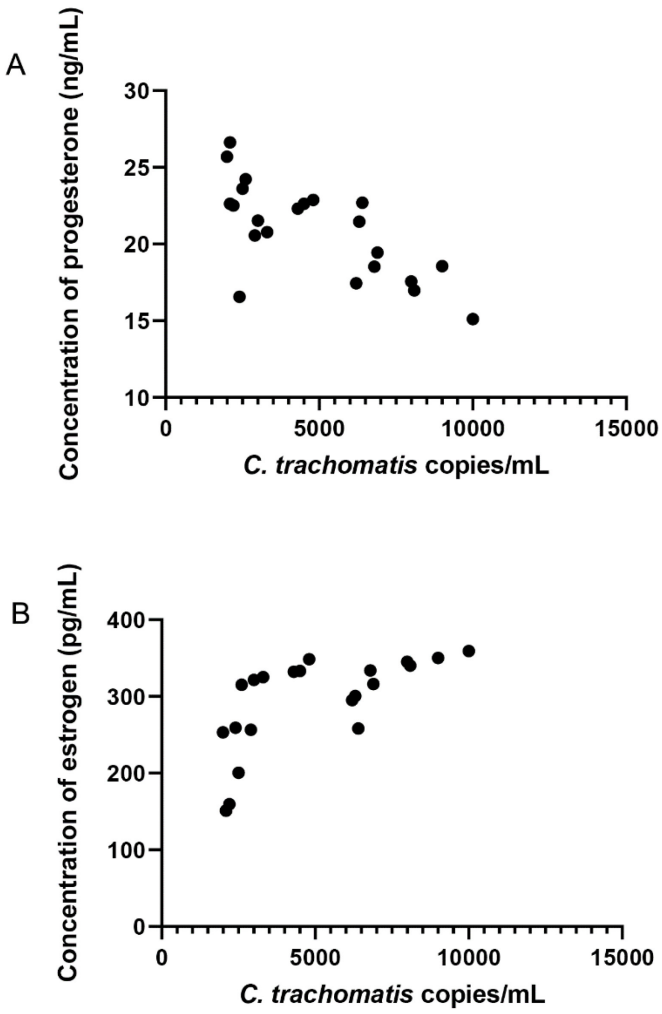
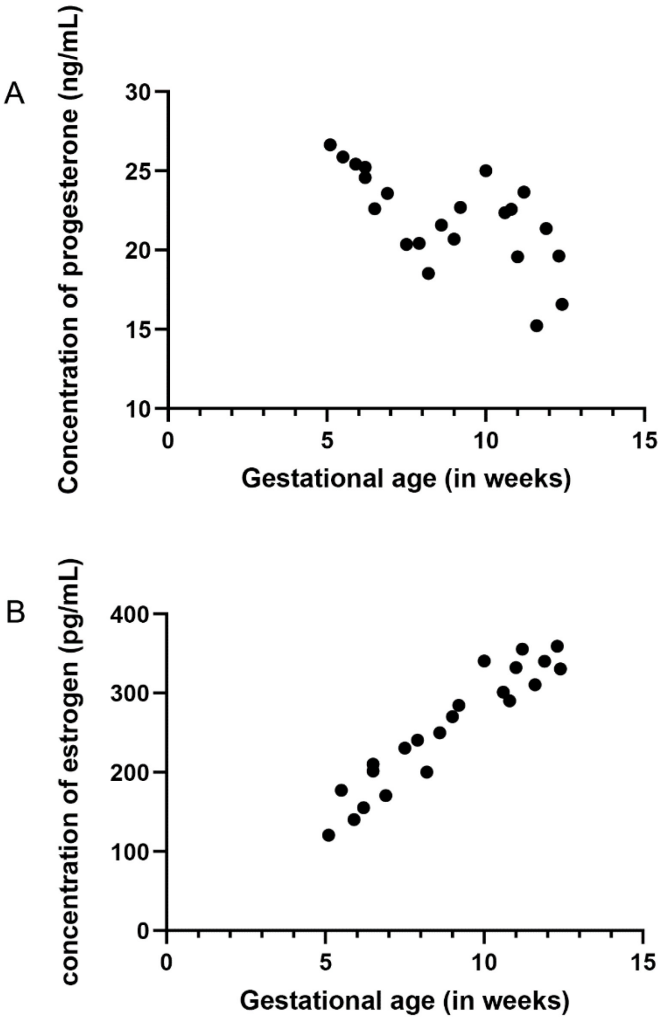


Figure 4. Correlation between **A:** concentration of progesterone and gestational age (in weeks); **B:** concentration of estrogen and gestational age (in weeks) (Spearman's rank correlation test).



proliferative phase, the endometrium is thin with low levels of estrogen and progesterone. As the proliferative phase ends, the follicles grow until ovulation is achieved which is indicated by increased concentration of progesterone (secreted by the corpus luteum) for maintenance of the endometrium. Thereafter, the corpus luteum regresses and menstruation takes place if conception has not taken place. At menstruation, serum estrogen levels are typically low, gradually increasing in the proliferative phase and dropping briefly at ovulation. Levels of estrogen and progesterone are present during the luteal/secretory phase with its highest levels at day 21 of a 28-day cycle. The level of estrogen returns to the menstrual levels toward the conclusion of the secretory phase [24].

For better management and intervention of female reproductive life, understanding of the mechanisms by which *C. trachomatis* cause significant disorders, particularly those impacting pregnancy outcomes, are required. *C. trachomatis* takes up a unique chronic course for establishment of pathology associated with inflammatory damage to the host [25]. A study reported low association of *C. trachomatis* in chorionic villi tissue of spontaneous miscarriage patients [26]. However, other previous/recent studies have demonstrated an association between RSA and *C. trachomatis* in various clinical samples such as endometrial curettage tissue and urine [3-6].

Both estrogen and progesterone are secreted by the ovaries and a well-developed placenta. These hormones play pivotal roles in the puberty period of adolescent females and are responsible for development of ovaries. Their inadequate secretion can lead to early abortion [27]. Studies in animal models have highlighted their importance during pregnancy. For instance, studies in mice and guinea pigs [28-29] show that progesterone as well as estrogen causes synthesis and release of prostaglandins from the uterine tissue. Serum levels of progesterone and estrogen are altered by lipopolysaccharides during preimplantation thereby elevating the estrogen/progesterone ratio in the mouse. It was further concluded by the authors that during bacterial infection, an altered ratio of the hormones during preimplantation resulted in failed pregnancy by reducing the uterine responsiveness [17]. Infection of ewes with *C. psittaci* caused late abortion due to elevation of circulating estrogens and prostaglandins and a decrease in progesterone [30]. This observation was also confirmed by other investigators [31]. Another study demonstrated a decrease in the cholesterol level in *C. trachomatis* infected trophoblast thereby depleting the levels of estrogen and progesterone suggesting diminished functions of trophoblast like implantation and thus affecting pregnancy [32].

Another study demonstrated that chlamydial infection decreased the level of specific progesterone markers responsible for embryo implantation with a decrease in the level of progesterone suggesting a possible mechanism by which *Chlamydia* might directly inhibit the effects of progesterone on uterine cells [32].

Our study demonstrated the possible effect of *C. trachomatis* infection on levels of progesterone/estrogen hormones and pregnancy outcome was observed to be decreased in infected patients while estrogen was increased when compared to controls suggesting that *C. trachomatis* inhibits the expression of progesterone thus inducing abortion [33]. A significant negative correlation between progesterone with chlamydial load clearly suggests that a high *C. trachomatis* infection load leads to decreased progesterone concentration. Also, a significant correlation was observed between the reproductive hormones and gestational age in the infected RSA patients. Our data shows that dysregulated expression of progesterone/estrogen with advanced gestational age might be a risk factor for abortion in *C. trachomatis*-infected patients. This study thus provides a preliminary basis of immune (hormonal) involvement with chlamydial infectious load/advanced gestational age in RSA patients. However, one limitation of the study is that the exact mechanism of *C. trachomatis* pathogenesis requires further elucidation.

Conclusions

This study concludes that chlamydial load influences the expression of the female sex hormones such as progesterone and estrogen during advanced gestational age in recurrent spontaneous aborters, thereby suggesting a probable mechanism by which *Chlamydia* induces RSA. Further investigations are, however, required to fully understand the underlying immune mechanism in RSA.

Acknowledgements

AR is grateful to University Grants Commission (UGC), GOI, New Delhi (India) for the award of Junior and Senior Research Fellowship.

Funding

The research work (Project ID2020-9453) was financially supported by Indian Council of Medical Research (ICMR), DHR, MoHFW (GOI), New Delhi (India).

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Conflict of interests: No conflict of interests is declared.