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Short Communication

Azithromycin Treatment Modulates Cytokine Production in *Chlamydia trachomatis* Infected Women

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Chlamydial infection of the lower genital tract usually spreads to the upper genital tract and is then responsible for more serious consequences of chlamydial infection, such as infertility, ectopic pregnancy, pelvic pain and pelvic inflammatory disease [1].

Genital infection with *Chlamydia trachomatis* and the resulting cytokine environment largely determines the outcome of infection and disease [2–7]. Chlamydial infections are usually treated with Azithromycin or Doxycycline [8]. The immunomodulatory effects of macrolides have mainly been shown *in vitro* or in animal experiments. It has been reported that macrolide antibiotics can interfere with cytokine production *in vitro* [9–11]. Azithromycin has also been reported to inhibit the production of pro-inflammatory cytokines [12–17]. There is little evidence from the clinical arena that these immunomodulatory actions of antibiotics play a role in terms of outcome. Therefore, further studies to investigate the potential usefulness of azithromycin are necessary to elucidate its mechanism of action. The direct relevance of these findings for the treatment of infectious and inflammatory diseases will have to be tested in patients.

The results of clinical trials have suggested that antibacterial activity of azithromycin is efficacious in the treatment of chlamydial cervical infection [18–20]. However, there are no human studies that have assessed antibacterial as well as anti-inflammatory effects of azithromycin treatment in different clinical conditions associated with the chlamydial infections. In a recent study on a Macaque model, azithromycin effectively prevented the progression of inflammation and fibrosis in both the lower and upper reproductive tract after repeated chlamydial infections suggesting that azithromycin may provide anti-inflammatory as well as antimicrobial properties and this combined activity may be particularly well-suited for the treatment of chlamydial infection [21]. Therefore, it might be possible that in immunopathological conditions like infertility and pelvic inflammatory disease, the anti-inflammatory property of azithromycin may act in the eradication of infection and disease. Hence, in the present study in order to examine the anti-inflammatory effects of azithromycin, we investigated the levels of interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) in women representing different clinical conditions, that is, *Chlamydia*-positive fertile and infertile women with genital tract infection of *C. trachomatis* before and after treatment with azithromycin.

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Materials and Methods

Study population. A total of 238 patients attending the gynaecology outpatient department, Safdarjung Hospital, New Delhi, India, for gynaecological complaints (cervical discharge, cervicitis and infertility) were enrolled in the study. The study received approval by the hospital's ethics review committee (Ethical Committee/9/2005/dated 10 June 2005). Thirty healthy age-matched controls attending the family-planning department for birth-control measures and with no previous history of any sexually transmitted disease were also enrolled. At recruitment, a detailed clinical questionnaire was administered to each patient for collecting information on reasons for referral, gynaecology history including menstruation, symptoms of genital and urinary tract infection, obstetric and medical histories. Patients with positive urine pregnancy test, recent antibiotic therapy and genital tuberculosis were excluded from the study. Male-related infertility cases were also excluded from the study.

Collection of samples. The vulva was examined for lesions and vaginal/cervical discharge. The cervix was inspected for ulcers, warts, ectopy, erythema, discharge or any other abnormalities.

A cotton tipped swab (Hi Media, Mumbai, India) after being introduced into the cervical canal through a Cusco's speculum, was rotated at 360° in the endocervical canal to remove mucoid discharge and then discarded. Following this, two swab samples were collected in phosphate-buffered saline for *C. trachomatis* detection by polymerase chain reaction and Direct Specimen Test. In addition, for diagnosis of sexually transmitted disease pathogens, viz. *Candida sps*., bacterial vaginosis, *Trichomonas vaginalis*, another swab was taken and smeared on glass slides. Furthermore, one swab sample was collected in phosphate-buffered saline for culture for detection of *Neisseria gonorrhoeae, Mycoplasma hominis*, *Ureaplasma urealyticum*. Cervical secretions were collected in 5 ml sterile saline administered through a sterile pasteur pipette and recovered by aspiration. It was further centrifuged at $200 \times g$ for 10 min. at 4°C and supernatant was aliquoted and stored at −80°C maximum for 15 days until assay after adding 0.5% (vol/vol) protease inhibitor cocktail (Sigma). Non-heparinized peripheral venous blood (2 ml) was also collected.

Cervical secretions were collected both pre- and post-azithromycin treatment during mid-cycle (median 14 days, ranged from 12th to 16th day for pre-treatment and median 13 days, ranged from 12th to 15th day for post-treatment) of the menstrual cycle.

Laboratory diagnosis. Spots were made on glass slides from cervical swab samples. These were stained with fluorescein isothiocyanate-conjugated monoclonal antibodies to *C. trachomatis* major outer membrane protein using *C. trachomatis* Direct Specimen Test kit (Microtrak, Syva Corporation, Palo Alto, CA, USA) according to the manufacturer's instructions. A sample was considered to be positive when at least 10 elementary bodies were detected. Samples with more than one and less than 10 elementary bodies were confirmed for positivity by polymerase chain reaction analysis using a primer specific for 200 base pair (bp) plasmid of *C. trachomatis* [22]. Diagnosis for other sexually transmitted disease pathogens were done by culture for *N. gonorrhoeae, M. hominis*, *U. urealyticum* and by microscopy on gram-stained smears for *Candida sps.*, bacterial vaginosis, *Trichomonas vaginalis* as mentioned earlier [23].

Treatment. After diagnosis, patients were prescribed azithromycin (1 g single dose) and samples were again collected after 4 weeks.

Quantification of Cytokines in cervical secretions and serum. Quantification of IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IFN-γ, and TNF-α in cervical secretions and serum was done by commercially available ELISA kits (eBiosciences, San Diego, USA) in accordance with the manufacturer's instructions.

Statistical analysis. Differences between two groups were evaluated using the Mann–Whitney *U* test. Wilcoxon signed rank test was used to compare cytokine concentrations before and after therapy.

Results

Study population. Cervical *C. trachomatis* infection was diagnosed by direct fluorescent assay /polymerase chain reaction in 115 patients. Thirty-eight of these patients were found to have bacterial vaginosis, or to be co-infected with either *Candida* sp., *T. vaginalis*, *M. hominis*, *U. urealyticum* or *N. gonorrhoeae* in the cervix and were thus excluded from the study. After treatment, two patients were found to be positive for *C. trachomatis* and were hence excluded from the study. Ten patients were excluded from the study as apart from azithromycin, other antibiotics were also prescribed to them. Twenty-seven patients did not turn up after treatment. Based on diagnosis, the women were divided into three groups. Group I (n = 30) comprised of uninfected healthy controls with no infertility problem; Group II (n = 20) comprised of *Chlamydia*-positive women with no infertility problem; Group III (n = 18) comprised of *Chlamydia*-positive women with infertility. Infertile patients had regular unprotected intercourse for at least 2 years without conception. The median ages of women in each group were comparable; Group I: 24 (21–41); Group II: 28 (20–45); and Group III: 26 (22–46).

Measurement of cytokines in cervical secretions and serum of patients and controls. In cervical secretions, IL-8 levels were significantly higher in Group II ($P = 0.0007$) and Group III ($P < 0.0001$), respectively, as compared to Group I. Significantly higher levels of IL-10 ($P = 0.03$), IFN-γ ($P = 0.009$), and TNF-α ($P = 0.02$) were observed in Group III as compared to Group I. The IFN-γ and TNF-α levels were higher but not significant in Group II as compared to Group I. Levels of IL-1β were higher but not significant in Group III as compared to Group I. No significant difference was observed in the levels of IL-2, IL-4, IL-6, and IL-13 in both the patients group (Group II and Group III) as compared to Group I (table 1). There was no detectable IL-4 level in any of the samples (data not shown). In serum, only IL-8 levels were significantly higher ($P = 0.005$) in Group III as compared to Group I. There was no significant difference in the serum levels of IL-1β, IL-2, IL-4, IL-6, IL-10, IL-13, IFN-γ, and TNF-α (table 2).

Measurement of cytokines in cervical secretions and serum of patients before and after azithromycin treatment. In cervical secretions, IL-8 ($P = 0.0001$ and 0.0003), IFN-γ ($P = 0.01$ and 0.01), and TNF-α ($P = 0.01$ and 0.01) levels were significantly decreased in both Group II and Group III, respectively, after treatment with azithromycin as compared to before treatment in the patient groups (table 3); whereas only IL-8 was significantly decreased ($P = 0.04$) in serum in Group III after treatment (table 4).

Discussion

The interference of macrolide antibiotics, with the natural effector molecules involved in antimicrobial defences and inflammation, is an area under active investigation. Such

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Table 1.

Cytokine concentration in cervical secretions.

	Group I (n = 30)	Group II (n = 20)	P value*	Group III (n = 18)	P value†
IL-1β (pg/ml)	6 (UDL-25)	6.5 (UDL-21)	0.6	14.5 (UDL-38)	0.06
IL-2 (pg/ml)	7 (UDL-18)	5.5 (UDL-17)	0.5	6 (UDL-32)	0.9
IL-6 (pg/ml)	4.5 (UDL-31)	3.5 (UDL-27)	0.4	4 (UDL-15)	0.5
IL-8 (pg/ml)	15.5 (UDL-50)	52 (7-179)	0.0007‡	69.5 (8-213)	<0.0001‡
IL-10 (pg/ml)	6 (UDL-20)	7 (UDL-28)	0.6	17 (UDL-40)	0.03‡
IL-13 (pg/ml)	5 (UDL-20)	4.5 (UDL-16)	0.6	5.5 (UDL-14)	0.8
IFN-γ (pg/ml)	10 (UDL-41)	31 (UDL-97)	0.1	59 (UDL-178)	0.009‡
TNF-α (pg/ml)	8 (UDL-36)	18.5 (UDL-79)	0.2	40 (UDL-116)	0.02‡

Group I comprised of uninfected healthy controls with no infertility problem; Group II comprised of *Chlamydia*-positive women with no infertility problem; Group III comprised of *Chlamydia*-positive women with infertility.
*Denotes significance level between Group II and Group I.
†Denotes significance level between Group III and Group I.
‡Denotes significance level.
Cytokine concentration is denoted by median and range in parenthesis.
UDL-Under detection limit.
Differences between two groups were evaluated using the Mann–Whitney *U* test.

research is part of the larger, complex investigation of the immunomodulatory activity of antimicrobial agents [9].

During chlamydial infection in animals and humans as well as in cell culture systems, a wide array of inflammatory cytokines is considered to contribute to *Chlamydia*-induced pathologies [2–7]. Hence, apart from antibacterial activity, its treatment may require modulatory effect on cytokines to clear the pathology associated with *C. trachomatis*. Therefore, in the present study, our aim was to elucidate if azithromycin also modulates the production of cytokines in the process of eradication of infection. We have found that after treatment with azithromycin in the patient groups, levels of IL-8, IFN-γ, and TNF-α decreased in cervical secretions. We have also found that serum IL-8 levels decreased after azithromycin treatment in the patients. Previous reports suggest that macrolides including erythromycin, clarithromycin, and azithromycin exhibit immunomodulatory activities, such as inhibition of neutrophil chemotaxis and oxidative burst and the release of pro-inflammatory cytokines from monocytes [9–11]. Azithromycin has also been reported to inhibit cytokines both *in vivo* in animal models and *in vitro* [12–17]. Consistent with these reports, we have also found that azithromycin inhibits cytokine production which is associated with pathological condition in *C. trachomatis*-infected fertile and infertile patients. Hence, this finding suggests that azithromycin anti-inflammatory activity may have clinical significance in treating chlamydial infection in an immuno-pathological condition like infertility.

Furthermore, we have observed that in cervical secretions (at the site of infection), maximum cytokine secretion was inhibited whereas in serum decrease in only IL-8 levels was observed after azithromycin treatment. It has been reported that azithromycin differs from other macrolide antibacterials in that it exhibits unusual pharmacokinetic properties and is

Table 2.

Cytokine concentration in serum.

	Group I (n = 30)	Group II (n = 20)	P value*	Group III (n = 18)	P value†
IL-1β (pg/ml)	5.5 (UDL-17)	4.5 (UDL-14)	0.4	4.5 (UDL-18)	0.6
IL-2 (pg/ml)	4.2 (UDL-6)	4.4 (UDL-8)	0.3	4 (UDL-8)	0.7
IL-6 (pg/ml)	3.3 (UDL-9)	3.7 (UDL-12)	0.6	4 (UDL-10)	0.3
IL-8 (pg/ml)	6.5 (UDL-48)	12.5 (UDL-94)	0.08	23 (3-142)	0.005‡
IL-10 (pg/ml)	3.8 (UDL-10)	4.4 (UDL-8)	0.4	3.6 (UDL-9)	0.2
IL-13 (pg/ml)	4.6 (UDL-10)	4.5 (UDL-11)	0.8	5 (UDL-8)	0.6
IFN-γ (pg/ml)	8 (UDL-24)	10 (UDL-28)	0.3	8 (UDL-18)	0.1
TNF-α (pg/ml)	6 (UDL-22)	8.5 (UDL-39)	0.3	6.8 (UDL-26)	0.2

Group I comprised of uninfected healthy controls with no infertility problem; Group II comprised of *Chlamydia*-positive women with no infertility problem; Group III comprised of *Chlamydia*-positive women with infertility.
*Denotes significance level between Group II and Group I.
†Denotes significance level between Group III and Group I.
‡Denotes significance level.
Cytokine concentration is denoted by median and range in parenthesis.
UDL, under detection limit.
Differences between two groups were evaluated using the Mann–Whitney *U* test.

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Table 3.

Cytokine concentration in cervical secretions before and after treatment.

	Group II (n = 20)			Group III (n = 18)		
	Before treatment	After treatment	P value	Before treatment	After treatment	P value
IL-1β (pg/ml)	6.5 (UDL-21)	7 (UDL-30)	0.2	14.5 (UDL-38)	11 (UDL-26)	0.3
IL-2 (pg/ml)	5.5 (UDL-17)	5 (UDL-22)	0.8	6 (UDL-32)	5 (UDL-12)	0.5
IL-6 (pg/ml)	3.5 (UDL-27)	4.5 (UDL-23)	0.9	4 (UDL-15)	4 (UDL-25)	0.4
IL-8 (pg/ml)	52 (7-179)	27 (5-88)	0.0001*	69.5 (8-213)	22.5 (7-114)	0.0003*
IL-10 (pg/ml)	7 (UDL-28)	8.5 (UDL-35)	0.3	17 (UDL-40)	18 (UDL-36)	0.4
IL-13 (pg/ml)	4.5 (UDL-16)	5 (UDL-18)	0.8	5.5 (UDL-14)	5.5 (UDL-23)	0.7
IFN-γ (pg/ml)	31 (UDL-97)	12.5 (UDL-64)	0.01*	59 (UDL-178)	21.5 (UDL-134)	0.01*
TNF-α (pg/ml)	18.5 (UDL-79)	8.5 (UDL-32)	0.01*	40 (UDL-116)	14.5 (UDL-56)	0.01*

Group II comprised of *Chlamydia*-positive women with no infertility problem; Group III comprised of *Chlamydia*-positive women with infertility.
*Denotes significance level.
Cytokine concentration is denoted by median and range in parenthesis.
UDL, under detection limit.
Wilcoxon's-signed rank test was used to compare cytokine concentrations before and after therapy.

Table 4.

Cytokine concentration in serum before and after treatment.

	Group II (n = 20)			Group III (n = 18)		
	Before treatment	After treatment	P value	Before treatment	After treatment	P value
IL-1β (pg/ml)	4.5 (UDL-14)	5.8 (UDL-20)	0.3	4.5 (UDL-18)	5 (UDL-15)	0.2
IL-2 (pg/ml)	4.4 (UDL-8)	5 (UDL-10)	0.5	4 (UDL-8)	4.6 (UDL-8)	0.3
IL-6 (pg/ml)	3.7 (UDL-12)	3 (UDL-14)	0.8	4 (UDL-10)	4.5 (UDL-12)	0.6
IL-8 (pg/ml)	12.5 (UDL-94)	8.5 (UDL-66)	0.1	23 (3-142)	17 (7-96)	0.04*
IL-10 (pg/ml)	4.4 (UDL-8)	3.5 (UDL-8)	0.5	3.6 (UDL-9)	4 (UDL-12)	0.6
IL-13 (pg/ml)	4.5 (UDL-11)	4.5 (UDL-12)	0.9	5 (UDL-8)	4.8 (UDL-10)	0.5
IFN-γ (pg/ml)	10 (UDL-28)	12 (UDL-30)	0.3	8 (UDL-18)	9 (UDL-34)	0.3
TNF-α (pg/ml)	8.5 (UDL-39)	6.5 (UDL-28)	0.5	6.8 (UDL-26)	8 (UDL-36)	0.4

Group II comprised of *Chlamydia*-positive women with no infertility problem; Group III comprised of *Chlamydia*-positive women with infertility.
*Denotes significance level.
Cytokine concentration is denoted by median and range in parenthesis.
UDL, under detection limit.
Wilcoxon's-signed rank test was used to compare cytokine concentrations before and after therapy.

rapidly accumulated by cells and tissues [24]. In addition, concentrations of azithromycin in tissue are much higher than are concentrations in serum [25]. Hence, it can be suggested that azithromycin may have maximum effect at the site of infection.

Overall, our data suggest that azithromycin may provide anti-inflammatory as well as antimicrobial properties which could contribute to its clinical efficacy suited for the treatment of chlamydial infection in immunopathological conditions like infertility. These results may further improve the understanding of chlamydial therapy and biology.

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