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Original Research Article

Antibiotic treatment of asymptomatic *Ureaplasma* infection improves semen parameters in infertile men

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ABSTRACT

The role of asymptomatic infections caused by *Ureaplasma* species in male infertility and the efficacy of antibiotics in treatment of this failure is not yet definitely determined. A total of 165 infertile males having abnormal semen parameters (study group) as well as 165 healthy fertile men (control group) were included in this study. Semen samples were taken from all participants and after analyzing, undergone real-time PCR, microbial culture, and reactive oxygen species (ROS) as well as total antioxidant capacity (TAC) assays. Infected individuals of study group were treated with antibiotic. One month after the treatment completion, second semen samples were taken and undergone all the tests mentioned. The frequency of *Ureaplasma* spp. was significantly higher in the infertile men compared with the fertile ones (36.4% versus 11.5%; $p < 0.001$). Most of semen parameters were improved ($p < 0.05$) and reached their normal range, the level of TAC elevated ($p < 0.001$), and ROS level ($p = 0.003$) as well as ROS/TAC ratio ($p = 0.003$) reduced after antibiotic treatment. Moreover, wives of 37 infertile men (61.7%) became pregnant six months after the treatment completion. These findings indicate that *Ureaplasma* species are correlated with male infertility and that antibiotic therapy can improve the semen parameters and treat the male infertility.
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Introduction

Ureaplasma species are considered among the most prevalent sexually transmitted pathogens that have a global distribution. They are usually found as part of the normal microbiome of the human urogenital tract (Razin 2006; Bharat et al., 2015). These bacteria are associated with some symptomatic and asymptomatic genitourinary tract infections in both males and females, such as non-gonococcal urethritis (NGU), endometritis, bacterial vaginosis, preterm delivery, postpartum or postabortal fever, pelvic inflammatory disease (PID), ectopic pregnancy, as well as perinatal disorders such as low birth weight and neonatal bacteremia/meningitis (Waites et al., 2005; Viscardi 2010; Taylor-Robinson and Lamont, 2011; Ahmadi et al., 2016).

Some investigators believe that Ureaplasmas could negatively change various semen parameters, such as sperm motility, count, and morphology, and/or cause oxidative damage to spermatozoa via producing of ROS and causing the imbalance between ROS and TAC in seminal fluid, thereby leading to male infertility (Smith et al., 1996; Xu et al., 1997; Nunez-Calonge et al., 1998; Sharma et al., 1999; Potts et al., 2000; Reichart et al., 2000; Han et al., 2003; Rybar et al., 2012; Lee et al., 2013; Huang et al., 2014; Zhang et al., 2014), while other researchers have reported no influence of Ureaplasmal infections on semen quality (Günyeli et al., 2011; La Vignera et al., 2011). However, the effect of urogenital *Ureaplasma* infections particularly asymptomatic ones on spermatozoa and seminological variables as well as their role in male or female infertility is still controversial and remains unclear (Potts et al., 2000; Knox et al., 2003; Sanocka-Maciejewska et al., 2005; Wang et al., 2006a; Ochsendorf, 2008).

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There are many clinically asymptomatic carriers silently colonized by these bacteria while these microorganisms are potentially pathogenic and may play a role in urogenital tract infection or affect fertility potential as an opportunistic pathogen, under certain circumstances (Klein et al., 1969; Pannekoek et al., 2000; Al-Daghistani and Abdel-Dayem, 2010; Günyeli et al., 2011; Salmeri et al., 2012; Ahmadi et al., 2016). Nevertheless, the majority of asymptomatic infections may remain undetected and consequently untreated (Ahmadi et al., 2016).

The aims of the present study were to: 1) compare the frequency of *Ureaplasma* species between the study group (clinically asymptomatic infertile men with abnormal semen parameters) and the control group (healthy fertile men with normal semen parameters), 2) elucidate the association of asymptomatic *Ureaplasma* infections with male infertility, 3) assess the semen level of ROS, TAC, and the ROS/TAC ratio in both study and control groups, and 4) evaluate the effect of antibiotic treatment on improvement of spermatozoa parameters, semen levels of ROS and TAC, and ROS/TAC ratio in infected infertile men.

Materials and methods

Patient enrolment

Ethics approval for this study was obtained from the Ethics Committee of Royan (No. IR.ACECR.ROYAN.REC.1395.52). The patients in this study were selected from men consecutively admitted to the Royan Institute for Reproductive Biomedicine, Tehran, Iran. All participants as well as their sexual partners provided written informed consent. All the patients were clinically examined and asked for past medical, sexual, and social histories.

Inclusion and exclusion criteria

Included patients (the study group) were those that had abnormal semen analysis results in which at least one semen parameter (sperm count, motility, or normal morphology) being below the latest reference value recommended by the World Health Organization (WHO) (World Health Organization, 1999). Patients displaying any symptoms of urogenital tract infections or having endocrine disorders, chromosomal anomalies, reproductive system abnormalities (varicocele, hydrocele, or undescended testis), testicular tumors, systemic diseases, sperm autoantibodies, or those who had history of antibiotic use within the previous week were not included in our study. Males with azoospermia, heavy use of alcohol, heavy smoking, continuous exposure to chemical or physical agents with known adverse reproductive effects (e.g., benzene and radiation), as well as patients whose semen samples tested positive for *Ureaplasma* spp. by real-time polymerase chain reaction (real-time PCR), but negative by microbial culture, were also excluded from the study.

In all, 165 clinically asymptomatic men with abnormal semen parameters having infertility of at least one-year duration fulfilled the eligibility criteria for inclusion in this study. In addition, 165 healthy fertile men with normal semen parameters attending for routine check-up were enrolled in a consecutive manner over the study period and designated as the control group. The female partners of all participants had normal results on fertility evaluation.

Semen collection and analysis

Given written and verbal instructions to the participants to follow the procedure, semen samples were collected into sterile sample cups through self-administered masturbation, after 3–7 days of sexual abstinence. Samples were put in the incubator directly for liquefaction and then manually analyzed by the same person for volume, viscosity, pH, presence of white blood cells (WBCs), sperm concentration (count/ml and total count), motility (classes A, B, A+B, C, and total), and normal morphology, as indicated by WHO manual for semen analysis (World Health Organization, 1999). Semen analysis was confirmed using a light microscope equipped with a Computer-Aided Semen Analysis (CASA; Test Sperm 2.1, Videotest, St. Petersburg, Russia) system.

Afterward, seminal fluids collected from study and control groups were divided to four parts for performing: 1) real-time PCR (200 μl), 2) microbial culture (500 μl, inoculated into Transport-PPLO broth medium), 3) ROS level assay (100–500 μl), and 4) TAC test (100–200 μl, stored at −70° C till time of the test).

DNA extraction

Ureaplasma DNA was extracted from semen samples using High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. Negative Control of Extraction was used in the extraction procedure and Internal Control, which serves as an amplification control, was directly added to the sample/lysis mixture during the DNA isolation for each individually processed specimen to identify possible reaction inhibition.

Real-time PCR

Ureaplasma species Real-TM commercial kit (Sacace Biotechnologies Srl, Como, Italy) was used for qualitative detection of *Ureaplasma* species. Reagent preparation was performed according to the manufacturer's instructions and real-time PCR was performed on a Rotor-Gene™ 6000 (Corbett Life Science, Sydney, Australia). The temperature profile of amplification was as follows: step 1) 95 °C for 15 min, followed by step 2) five cycles of 95 °C for 5 s, 60 °C for 20 s and 72 °C for 15 s, and step 3) the last step of 40 cycles of 95 °C for 5 s, 60 °C for 20 s and 72 °C for 15 s. Fluorescent signal detection was accrued at the second stage of the step 3 (60 °C). Amplification data were analyzed by the instrument software (Rotor-Gene software series, version 1.7), and cycle threshold (C_t) values ≤33 were considered positive.

Microbial culture for Ureaplasma *spp. detection*

Semen samples found positive in real-time PCR, underwent microbial culture in order to ensure that the detected bacteria were viable and could grow and form the colonies.

Media for *Ureaplasma* culture and isolation including Transport-PPLO broth, Urea-PPLO broth, and Urea-PPLO agar, were prepared using a commercial PPLO Broth/Agar Base Without Crystal Violet (Conda, Madrid, Spain). Supplements and additives including 10% Yeast extract (Conda, Madrid, Spain), 20% horse serum (Baharafshan, Tehran, Iran), 0.1% urea (Carlo Erba, Italy), 1000 IU/ml penicillin G, 0.002% phenol red (Sigma, St. Louis, MO, USA) as a pH indicator, and 0.01% $MnSO_4$ solution (for agar medium), were added to the suspension, and final pH was adjusted to 6.0 for Urea-PPLO broth/agar, and to 7.0 for Transport-PPLO broth (Tully and Razin, 1983).

Seminal fluid (500 μl) inoculated into Transport-PPLO broth medium, was immediately transported to microbiology laboratory, where the medium was passed through a 0.45 μm pore size disposable syringe filter (Minisart, Sartorius, Goettingen, Germany) and inoculated into Urea-PPLO broth medium. The latter medium was incubated aerobically at 35–37 °C. The tubes were hold for seven days and inspected once daily for color changes in the broth. As soon as a noticeable alkaline shift in pH (yellow to orange-red) was observed, the broth was immediately

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