



CYTOKINE PROFILES AMONG ASTHENOSPERMIC MEN WITH *CHLAMYDIA TRACHOMATIS* INFECTIONS: CONCENTRATIONS AND SIGNIFICANCE OF MULTIPLEX SEMINAL FLUID CYTOKINE AND OTHER IMMUNOLOGIC FACTORS

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ABSTRACT

Evaluation the role of interleukin-1 (IL- 1), interleukin-8 (IL- 8) and tumor necrosis factor alpha (TNF) in asthenospermic causes male infertility. In addition to revealed the prevalence of infection with *Chlamydia trachomatis*. Interleukin 1, IL-8 and Tumor Necrosis Factor Alpha levels were measured in seminal plasma of in different groups of infertile males as well as in control men, using PCR technique to detect the infection of *Chlamydia trachomatis* by detection of KL gene (241bp). In addition to detect STS polymorphisms in Y-Chromosome by multiplex PCR procedure. The mean \pm SE of the cytokines for Normospermia IL-1 (24.4 ± 0.8), IL-8 (134.6 ± 15.4), TNF- (7.14 ± 0.34). While the mean of the cytokines for asthenospermic IL-1 (48.8 ± 0.87), IL-8 (365.1 ± 8.8), and TNF- (32.9 ± 1.186). There was significantly elevated in the levels of TNF and Interleukin 1 in seminal fluid correlate with leukocyte counts and ratios in the same ejaculates, also there was significantly elevated in the levels of IL-8 in seminal levels among infertile groups compared to normal control subjects. Prevalence of *C. trachomatis*: (23.38%) of the sample from the Asthenospermic group gave 241 bp DNA band in agarose gel electrophoresis, in comparison to 5.19% among the control group. Cytokines and especially IL-1, 8 and TNF may play a role in pathogenesis of male factor infertility and may be a part of infertility workup in near future. *C. trachomatis* infection was elevated among asthenospermic men compared with normospermic men.

KEY WORDS: Cytokine, IL-1, IL-8, TNF- , *Chlamydia trachomatis*, Asthenospermia, Y-Chromosome microdeletion, PCR

INTRODUCTION

It is estimated that approximately 2–6% of men who undergo a vasectomy for sterilization ultimately want to restore their fertility and request to have the procedure reversed. Patency rates after vasectomy reversal range from 30% to 97%. However, up to 72% of men remain infertile. The reason for this is unclear. Infertility^[1] is a problem of global proportions, affecting on average 8-12 percent of couples worldwide^[2]. The major cause of infertility in Africa is infection-sexually transmitted diseases, post-aborted and puerperal sepsis^[3]. The prevalence of male accessory gland infection varies widely in different regions of the world, it is generally accepted that asymptomatic carriage and transmission may be of significance. Infectious processes may lead to deterioration of spermatogenesis, impairment of sperm function and/or obstruction of the genital tract^[4]. Sexually transmitted infections (STI) are a major public health problem throughout the world^[5]. STI can affect fertility by destroying sperm function or by obstructing seminal tract^[6]. Human papillomavirus (HPV) is a small DNA virus that is responsible for fastest spreading STI in humans. The vast majority of sexually active men and women will become infected with HPV in their lives. Currently, there are neither good methods for preventing, nor comprehensive and effective treatments for the clinical consequences of HPV infection. Fortunately, the majority

of those infected with HPV will not develop clinical disease or symptoms because the host immune system resolves most infections^[7]. *Chlamydia trachomatis* is the main bacterial agent in the etiology of STI in sexually active men and women. *C. trachomatis* infection is also associated with more persistent HPV infection, which may contribute to the increased risk of clinical complications of HPV in individuals co-infected with *C. trachomatis*^[8]. The survival and function of the gametes, protection and proliferation of the embryo during its migration through the uterus, as well as its subsequent development, depend on local regulation mechanism (hormones and growth factors). Recently investigation pointed out the importance of the small soluble polypeptide mediators, cytokines, in various aspects of reproduction^[9, 10]. Measuring the levels of cytokines, in seminal plasma, does not only expand the diagnostic option, but also, through the growing knowledge of immune process, can give rise to new therapeutic methods of improving the quality of semen and increasing the chance to reproduce^[11]. Cytokines, important intracellular communicators, are involved in numerous physiological and pathological processes, which include mediation of inflammatory responses, reproductive physiology and regulation of gonadal steroid production and release^[12]. Human sperm contains a wide spectrum of cytokines such as tumor necrosis factor alpha (TNF) and interleukin 18 (IL-18)^[13]. There is clear

evidence indicating the effects of cytokines on spermatozoal functions. It has been found that TNF decreases the sperm motility^[14]. And stimulate sperm membrane lipid peroxidation by increasing reactive oxygen species generation^[15]. The initiator of infection is pathogen which may originated from either the urinary tract or be sexually transmitted. This has long been known, and among urinary invaders are usual pathogens are commonly found; *E. coli*, *Proteus*, *klebsiella* *Pseudomonas*, *Streptococcus* etc^[16]. There is clear evidence indicating the effects of cytokines on spermatozoa functions. It has been found that:

- i) IL-1 alpha, IL-1 beta and tumor necrosis factor-alpha (TNF-), stimulate sperm peroxidation by increasing ROS generation^[17];
- ii) There is a positive correlation between IL-6 levels in seminal plasma and membrane sperm lipid peroxidation^[18];
- iii) Interferon-gamma (IFN-) and TNF- have been shown to decrease the motility of spermatozoa^[19,20].
- iv) infertile patients with varicocele show elevated levels of IL-6 and ROS, and decreased levels of total antioxidant capacity^[21]
- v) Mean levels of IL-6, IL-8 and IL-11 are higher in the seminal plasma of patients with genital infection and oligoteratoasthenozoospermia than those in seminal plasma of normal fertile men^[22].

MATERIAL & METHODS

Patients

A total of 77 chosen males of infertile couples, who presented for infertility investigation were enrolled in this study. Some of the patients had clinical signs of genital tract infection and, apart from their infertility problem. The median age of the male patients was 34 (range 20-49) years. Primary infertility was found in 16 % of patients. Some of the patients had clinical signs of genital tract infection apart from their infertility problem. None of the patients was treated with antibiotics and corticosteroids during the time of the study,

Preparation of sperm

Fresh semen samples were obtained from healthy normozoospermic volunteers and Asthenospermic patients at the Laboratory of fertility unite / Babylon Maternity and

Children Hospital, by masturbation after a minimum of 3 days of sexual abstinence. Informed consent was obtained from all subjects. The semen samples were processed by Isolate density centrifugation gradient (two layers: 40% and 90%). The resulting pellet was re-suspended in original Biggers and Whittingham's medium, washed by centrifugation (300 × g for 10 min) and diluted to give 10 million spermatozoa/ml. This final sample was evaluated by the peroxidase test to guarantee the absence of leucocytes.

Detection of cytokines and immune factors

Detection and quantitation of the various cytokines and other immunologic factors in aliquots of thawed seminal plasma were accomplished using commercially available enzyme-linked immunosorbent assay (ELISA) kits (DRG, Germany) following the manufacturers' protocols.

Samples preparation for PCR:

DNA extraction procedure using one hundred µl of semen added to 50µl of an extraction buffer (10mmol/L Tris-HCL,pH 8.3; 50 mmol/L KCL,2.5mmol/L MgCL2, 0.45% Tween 20, and proteinase K at 100µg/mL (Sacace, Italy, DNA-Sorb-B extraction DNA from whole blood, plasma, semen, liquor, sputum, biopats, fecal extract, etc.). The mixture was homogenized using a vortex mixer for 10 seconds and then incubated for 60 minutes at 56 C and for 10 minutes at 95C. The mixture was centrifuged briefly, and the supernatant was collected and used directly for amplification without purification. The samples were maintained at -20 °C, until used.

PCR for detection of *Chlamydia trachomatis*:

The primers KL1- 2 were used to amplify a 241 bp fragment of chlamydial plasmid. A distinctive reaction protocol comprising a final volume of 50 µl, was composed of 5 µl of the DNA sample; 25 mM of MgCl₂; 25 mM dNTP; 1mM of each primer and 1.5U of Taq polymerase. Amplification conditions of PCR was done by using 30 cycles program: denaturation at 94°C for 90 second, then followed by annealing at 63°C for 1 minute and polymerization at 73°C for 80 second, followed by a final PCR extension at 72°C for 7 minutes. The products were analyzed by electrophoresis in a 1% agarose gel with ethidium bromide. The sequences of the forward primers (F) and reverse primers (R), and the size of the amplified fragment were illustrated in table A:

TABLE A: Synthetic oligonucleotides used in PCR

Primer	Nucleotide sequence (5' to 3')	Size (bp)
KL		241 bp
<i>KL1(F)</i>	TCCGGAGCGAGTTACGAAGA	
<i>KL2(R)</i>	AATCAATGCCCGGGATTGGT	

Experimental protocol

Aliquots of sperm were cured for 2 hr. at 37°C by the tested different cytokines at different concentrations, denominated here as physiological and infection inflammation concentrations, which were determined as the mean of several values taken from several authors^[23]. The physiological concentrations used were: IFN- (127.1 pg/mL), TNF- (1.6 pg/mL) and IL-6 (25.0 pg/mL). The infection-inflammation concentrations were:

IFN- (508.5 pg/mL) and TNF- (17.0 pg/mL) and IL-6 (125.0 pg/mL). In a second group of experiments, similar incubations were carried out, but now in the presence of leukocytes (5 × 10⁶/mL). For the first group, the control consisted of sperm cells in culture medium; for the second group, the control consisted of sperm cells in culture medium plus the indicated amount of leukocytes.

DNA analysis for Y-Chromosome microdeletion

In total, 77 patients were analyzed for the presence of microdeletions in the AZF region. Samples comprised isolated DNA from the patient's peripheral blood lymphocytes (native or frozen, in 0.5 ml 0.5 M EDTA solutions). DNA was isolated using a commercial set Promega DNA Kit (USA). PCR amplification was performed on the thermal cycler (smart cycler, USA). We performed either the duplex PCR with two fragments or two multiplex PCR with five fragments. Analysis was always performed with a male control sample, a female control sample and a blank sample. Negative results were confirmed only after three amplification failures. PCR products were separated electrophoretically in 2% agarose

gel at laboratory temperature, in TBE buffer at 100 mA for 50 min. DNA was visualized by EtBr, Which was added directly to the gel (0.5µg/µl) with the following evaluation on a UV transilluminator. A commercially provided synthetic 100bp marker was used as a DNA molecular weight standard.

RESULTS & DISCUSSION

This prospective study included 77 men from sub fertile men (Asthenospermic) with and without symptoms of genital tract infections. Semen samples were collected from out clinic and fertility clinic at Babylon maternity and children hospital, as shown in Table 1.

TABLE 1: showing the mean levels \pm SE of IL-1 (pg/ml), IL-8 (pg/ml) and TNF (pg/ml) in seminal plasma of all studied groups.

cytokines	Mean \pm SE of concentration ((pg/ml))	
	Asthenospermia	Normospermia
IL-1	48.8 \pm 0.87	24.4 \pm 0.8
IL-8	365.1 \pm 8.8	134.6 \pm 15.4
TNF-	32.9 \pm 1.186	7.14 \pm 0.34

The tested cytokines, IL-1, IL- 1 and TNF- , when present in the incubation medium at physiological concentrations were able to significantly raise the level of lipid peroxidation of the sperm membranes. When utilized at infection-inflammation concentrations, none of these cytokines was able to further raise the level of the membrane lipid peroxidation. TNF- showed a higher effect at infection/inflammation concentrations than at physiological concentrations. At infection/inflammation concentrations, TNF- and IL-1 produced a higher effect in the presence of leukocytes than in their absence. Since the article by Rasmussen *et al.*, 1997 ^[24], others have confirmed the elicitation of inflammatory mediators from nonimmune host cells infected in vitro with *C. trachomatis*. Infection of genital tract tissue cultures results in epithelial cell release of IL-1 and cellular damage, independent of inflammatory cell influx. The addition of IL-1 receptor antagonist to the cultures completely eliminates tissue destruction induced by infection, indicating a direct role for this cytokine in pathogenesis ^[25]. In vitro infection of genital tract epithelium tissue also results in the production of tumor necrosis factor (TNF) and increased expression of adhesion molecules on endothelial cells ^[26]. This milieu could easily lead to activation and recruitment of first innate and, later, adaptive immune cells to effect resolution of infection. However, subsets of these responses may also induce collateral damage to genital tract tissue.

Proinflammatory chemokines and cytokines have been documented in murine and guinea pig models of genital tract chlamydial infection. The up-regulation of integrins in the murine genital tract is coincident with the onset of infection. Detection of the neutrophil chemokine macrophage inflammatory protein-2 (MIP-2), a chemokine analogous to IL-8 in humans, in genital tract secretions of infected mice coincides with a rapid influx of neutrophils into the lower genital tract. Thus, data to date suggest that the inflammatory response to chlamydia begins and is

sustained by actively infected nonimmune host epithelial cells. Defining the specific responses that promote tissue damage and differentiating them from those that lead to benign resolution of infection is an important ongoing research goal ^[27].

Prevalence of *C. trachomatis*: (23.38%) of the sample from the Asthenospermic group gave 241 bp DNA band in agarose gel electrophoresis, in comparison to 5.19% among the control group. Sexually transmitted *Chlamydia trachomatis* infection was widespread public health concern because of its prevalence and potentially devastating reproductive consequences, including pelvic inflammatory disease (PID), infertility, and ectopic pregnancy. Although the pathologic consequences of infection are well established, the mechanisms of chlamydia-induced tissue damage are not fully understood. Histological examination of tissue samples from women with PID caused by *C. trachomatis* revealed neutrophils in endometrial surface epithelium and in gland lumens, dense sub epithelial stromal lymphocytic infiltration, stromal plasma cells, and germinal centers containing transformed lymphocytes ^[28]. The prominence of both neutrophils and chronic inflammatory cells in infected human female genital tract tissue samples does not assist in the determination of specific responses responsible for disease sequel.

Infection of non-immune host epithelial cells and resident tissue innate immune cells with chlamydia results in production of proinflammatory cytokines and chemokines that lead to recruitment and activation of first innate and, later, adaptive immune cells to effect resolution of infection; subsets of these responses induce collateral genital tract tissue damage. So, as a result, Infection of reproductive tract epithelium results in production of interleukin (IL)-1, tumor necrosis factor (TNF), IL-8 ^[29]. There are no chromosomal abnormalities distinguished in all examined metaphases from asthenospermic samples. FSH elevations were detected in significant levels in asthenospermic (14.2+1.64 mIU/l) while the concentration

of testosterone revealed normal values. Asthenospermic men have shown deletions in their Y chromosome as

mentioned in table 2. AZF deletions are deletions of the euchromatine part of the long arm of the Y chromosome.

TABLE 2: PCR Amplification Product Profile for AZF (STS for AZF factors) related to Asthenospermic and Normospermic.

Reagon	Gene loci	Normo. %	Astheno. %
AZF a	DYS148	4.49275	10.7143
AZF b	SY134	5.07246	7.14286
	SY149	4.63768	7.14286
	SYPR3	3.33333	5.35714
AZF c	SY158	6.37681	16.0714
	DAZ	7.10145	1.14286

TABLE 3: showing the mean levels \pm SE of physiological parameters in semen of all studied groups

Variable	Mean \pm SE	
	Asthenospermia	Normospermia
Volume	2.45 \pm 1.2	3.17 \pm 1.0
Rapid (a)	6.53 \pm 6.8	67.222 \pm 9.4
Slow (b)	9.92 \pm 8.1	19.069 \pm 5.1
Not (c)	18.49 \pm 9.0	8.31 \pm 4.4
Immotile (d)	63.85 \pm 17.8	5.39 \pm 3.0
Normal morph	15.36 \pm 6.0	83.89 \pm 3.4
Abnormal morph	83.31 \pm 1.3	16.1 \pm 3.4
Sperm concentration ($10^6/ml$)	11.66 \pm 8.1	34.14 \pm 7.7

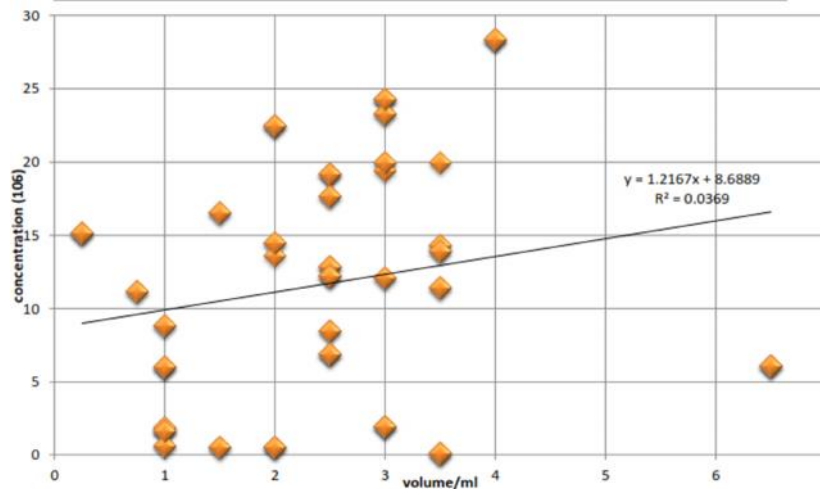


FIGURE 1: Correlation analysis between sperm concentration and volume among Asthenospermic patients

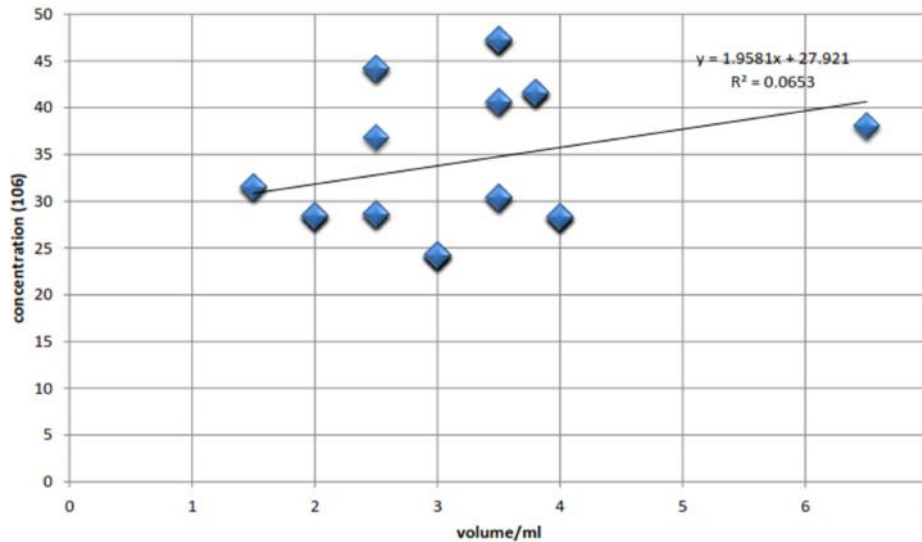


FIGURE 2: Correlation analysis between sperm concentration and volume among Normospermic patients

It is assumed that these deletions directly damage genes in this region that was responsible for the proper course of spermatogenesis. Y-chromosomal microdeletions are the second most frequent disorder of spermatogenesis in men with fertility disorder after Klinefelter's syndrome^[30]. Microdeletions in the AZF region are frequently founding patients with azoospermia. The incidence of these microdeletions has been found from 3 to 55%^[31]. EAA and EMQN published the laboratory guidelines for molecular analysis of Y-chromosomal microdeletions¹⁴. Therefore we decided to use six sequences and proceed in accordance with European guidelines in which all three sub-regions were represented by Y sequences: DYS148, SY134, SY149, SYPR3, SY158 and DAZ.

By using the set of sequences mention above revealed deletions in subregions AZFa, AZFb and AZFc, but there are no complete deletions of the whole AZF region. In the AZFa region we observed a deletion of sequence sY148 even in 10.7% cases of asthenospermic men. To confirm whether it is a partial deletion of the region and where the breakpoints are, a more detailed analysis of the particular region would be needed. We confirmed a deletion of the AZFc subregion represented by sequences sY158 and DAZ in 7.1% of patients. Further experience would be interesting particularly in the group of patients with various forms of asthenospermia.

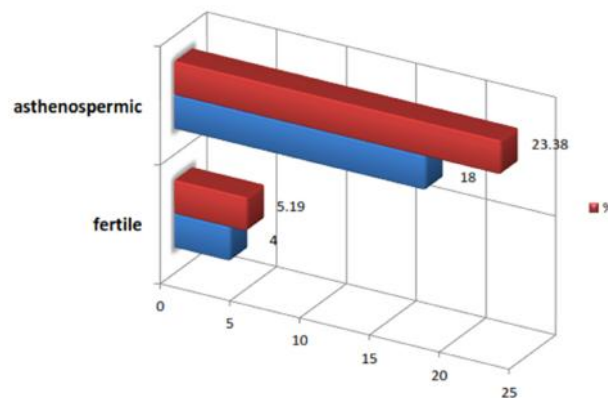


FIGURE 2: Prevalence of *C. trachomatis* infection among fertile (Normospermic) and unfertile (Anthenospermic) men

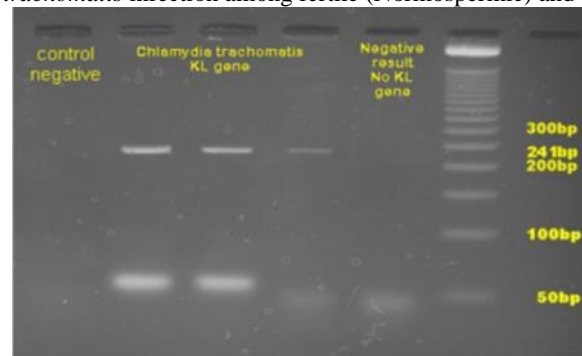


FIGURE 4: *C. trachomatis* kl gene (241bp). The ladder (L) lanes contain the 100bp DNA Step Ladder. 1% agarose gel in 1X TBE buffer containing 0.5 µg/ml ethidium bromide

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