Seminal Plasma Level of Interleukin-8 (IL-8) in Infertile Men With Silent Genital Tract Infection

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Aim : To confirm the presence of IL-8 in human seminal plasma, show differences between IL-8 concentrations in fertile and infertile subjects, and investigate the potential relationship between IL-8 concentration in seminal plasma and silent genital tract infection. Patients and Methods : A total of 20 chosen infertile, and 10 fertile men with no clinical signs of genital tract infection were investigated for basic infertility tests , andrological examination, seminal leukocyte count, seminal plasma IL-8 concentration by ELISA. For microbial screening, semen from cases and controls were cultured aerobically, detection of *C.trachomatis* and *M.genitalium* were done by PCR. Result: We detected statistically significant difference (*P<0.5*) of IL-8 in seminal fluid between fertile and infertile men, but smong the infertile men the concentration of IL-8 in seminal fluid was not significantly correlated with leukospermia (correlation coefficient =0.252) , nor with fertile group (correlation coefficient = -126), also no significantly correlation was detected between IL-8 concentrations and different semen testing including sperm count ,motility in both studied group. Semen cultures revealed aerobic growth in 33% of ejaculates, with potentially pathogenic species in 21% of these specimens ,statistically significant result was detected between aerobic culture and pus cells count (correlation coefficient =0.553). By PCR, 30%of the sample from the infertile group were positive for *C.trachomatis* in comparison to 5% among the control group, while *M. genitalium* was found in 20% of the infertile group compared to 5% in the control group. Positive aerobic cultures were not related to IL-8 concentrations in seminal fluid with correlation coefficient =.096. While IL-8 concentration give significantly correlation with *C.trachomatis* detection (0. 592). Conclusion: IL-8 concentration is associated with infertility and may be of importance in diagnosis of male infertility. IL-8 concentration can't be used as a marker for diagnosis of silent genital tract infection among infertile men, nor associated with seminal leukocytes count and other potential inflammation markers, but IL8 concentration significant correlate with *C.trachomatis* detection.

INTRODUCTION

Infertility is a problem of global proportions, affecting on average 8-12 percent of couples worldwide [1]. The major cause of infertility in Africa is infection-sexually transmitted diseases, post-abortal and puerperal sepsis [2].

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 [3] .

The clinical relevance of silent infection in asymptomatic patients is, however, not clear. Moreover, the interpretation of the markers commonly used for its diagnosis is controversial; for example the role of seminal leukocytes and clinically significant thresholds [4] .

During genital infection cytokines and various soluble receptors of immunoregulatory cytokines are expressed distinctly in seminal plasma. These factors also may be involved in the regulation of sperm cell functions and thus may affect male fertility [5].

Measuring the level of cytokines, both in seminal plasma and serum, does not only expand the diagnostic options, but also, through the growing knowledge of immune processes, can give rise to new therapeutic methods of improving the quality of semen and increasing the chance to reproduce [6].

Interleukin (IL) -8 is a potent neutrophil chemotactic and activating factor. It exerts its biological activities by binding to specific cell surface receptors. IL-8 may be involved, within a network of other cytokines, in intratesticular signal transduction, and may also adversely affect sperm membrane properties [7].

IL-8 concentrations may fluctuate during certain pathological conditions. There is little information about IL-8 interference

with sperm production and semen quality, and whether it increases in parallel to leukocyte counts and other potential markers of subclinical infection/inflammation in the same ejaculates, potentially interrelated with other cytokines. Also it is known whether potentially pathogenic microorganisms in the semen of patients, who are without symptoms of genital tract infection, are associated with increased pro-inflammatory seminal cytokine concentrations, which might impair sperm functional capacity [8] .

The aim of this study was to confirm the presence of IL-8 in human seminal plasma, investigate the differences between IL-8 concentrations in fertile and infertile subjects, and to show the potential relationship between IL-8 concentrations in semen and spermiogram parameters among the studied patient with silent genital tract infection in our locality.

SUBJECTS AND METHODS

Patients

A total of 20 chosen males of infertile couples, who presented for infertility investigation were enrolled in this study. None of the patients had clinical signs of genital tract infection and, apart from their infertility problem, were healthy individuals. The median age of the male patients was 33(range 21-53) years . Primary infertility was found in 76 % of patients, and secondary infertility in 24 %. The median duration of infertility was 4 years (range1-15) years. None of the patients had clinical signs of genital tract infection apart from their infertility problem, and were therefore considered asymptomatic of sexually transmitted disease they were healthy individuals. During the time of the study, none of the patients was treated with antibiotics, corticosteroids or antiphlogistics.

Fertile controls

Semen samples were analysed from 10 fertile men attending the Antenatal Care programme at the local health care centres caring for women in the same region as the women attending the gynaecological outpatient clinic The control group are similar in age and demographic characters of the patients

Basic infertility investigation and andrological examination

Standard semen analysis according to World Health Organization criteria [9] included determination of volume, pH, count, progressive motility after liquefaction (at room temperature), after 2 h and 4 h, morphology. Ejaculates were obtained in the lab. after at least 3 days of sexual abstinence, and were examined directly after liquefaction. Male infertility was classified according to WHO criteria .A detailed medical history was obtained and physical examinations were performed on both partners.

Laboratory investigation:

1- Seminal leukocyte count

After 3 days of ejaculatory abstinence, an ejaculate was collected in a dry plastic container . Sperm count was performed upon receipt of the sample, by use of a counting chamber as described by **Wolff,** [10] . Total and differential leukocyte counts were performed in 20 microscopy fields (×40) in a Papinocolaou-stained seminal smear from each sample. The concentration of leukocytes was expressed as millions of cells per milliliter of seminal fluid, calculated from their incidence relative to the average number of observed spermatozoa in the microscopy fields and with reference to the sperm count value. With regard to the number of leukocytes, samples with $>1x10^6$/ml leukocytes were considered as leukocytospermic, according to WHO definition[9]**.**

2- Seminal plasma IL-8 detection

The R.D International, Inc. (Minneapolis, MN 55413, USA) ELISA kit was used. One hundred ul of polyclonal antibody against IL-8 conjugated to HRP enzyme were added to each well containing standard or semen and incubated for 2.5 hours at room temperature. The wells were aspirated and washed with wash solution. Substrate solution (200 ul) were added and incubated for 30 minutes and 50 ul stop solution were added to all the wells. Optical density was measured by ELISA reader at 450 nm and after creating a standard curve, IL-8 levels were calculated[11] **.**

3- Microbial screening

To check semen samples for colonizing microorganisms, semen from cases and controls were cultured and detection of *C.trachomatis* and, *M.genitalium* was done by polymerase chain reaction (PCR)

Bacterial culture

An aliquot from all samples were cultured aerobically after a 1:2 dilution in saline solution. Standard bacteriological methods were used to quantify and identify all organisms according to previously published methods [12] .

Samples preparation for PCR:

A rapid DNA extraction procedure was used. One hundred µl of semen was 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 (Boehringer Mannheim, Mannheim, Germany). 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[13]

PCR for detection of Chlamydia trachomatis:

Amplification Reaction. The primers KL1- KL2 were used to amplify a chlamydial plasmid 241 bp fragment . A typical reaction system containing a final volume of 50 ml, was composed of 5 ml of the DNA sample; 25 mM of MgCl2; 25 mM dNTP; 1mM of each primer and 1.5U of Taq polymerase.

PCR amplification conditions :were done by using 35 cycles program: denaturation at 93°C for 1 minute, annealing at 64°C for 1 minute and polymerization at 72°C for 1 minute, followed by a final PCR extension at 72°C for 5 minutes. The products were analyzed by electrophoresis in a 2% agarose gel [14] .

PCR for detection of Mycoplasma genitalium:

The PCR target was a 495-bp fragment beginning 85 bp upstream from the M. genitalium adhesion gene start codon. The primer designations were MgPaW1 and MgPaWR1. The 25-L PCR reaction mixture contained 5 L of lysate, 25 pM of each primer, 0.25 mM of each dNTP (New England Biolab), 3.5 mM of MgCl2, and 2.5 U of Taq polymerase. PCR amplification conditions were 95°C for 10 min, followed by 35 cycles at 94°C for 40 s, 41°C for 40 s, 72°C for 40 s, and a final extension period of 15 min at 72°C. Ten microliters of the PCR product was electrophoresed through 1.2% agarose gel, stained with ethidium bromide,[15].

For both PCR experiment examination under UV light using the transilluminator FBTIV-88 (from Fisher scientific Pittsburg USA.) and gels containing bands were pictured using the built-in-Polaroid camera(Photo-Documentation, Hood FB-PDH-1216 .

The sequences of the forward primers (FP) and reverse primers (RP), and the size of the amplified fragment were illustrated in table(1) :

Table (1) Synthetic oligonucleotides used in PCR

Primer	**Size (bp)**	**Nucleotide sequence (5' to 3')**
KL1	241 bp	TCCGGAGCGAGTTACGAAGA
KL2		AATCAATGCCCGGGATTGGT
MgPaW1	495 bp	AAGTGGAGCGATCATTACTAAC
MgPaWR1		CCGTTGTTATCATACCTTCTGA

4- Statistical analysis:

It was performed by using spss program (**s**tatistical package of social science version 10) and EPi info prpgramm for WHO on windows 98. Mean ± SD, frequency and proportion were used . A 2-tailed Student's *t* test was used to compare 2 groups in quantitative data mean ± SD. Chi-square test was used for qualitative data (frequency & proportion),Odd ratio was calculated to

determine risk factors. A *P* value of ≤0.05 was considered to be statistically significant at Confidence interval 95%.

RESULT

IL-8 determination in seminal plasma

We detect significant difference of IL-8 in seminal fluid between fertile and infertile men, as the median concentration was 40 pg/ml and 125 respectively with statistically significant difference (*P<0.5*) .

IL-8 detection and result of semen analysis

Among , the infertile men the concentration of IL-8 in seminal fluid was not significantly correlated with the pus cells with correlation coefficient =0.252,nor with fertile group (-126). Also no significantly correlation was detected between IL-8 concentrations and different semen testing including sperm count ,motility in both studied group as shown in table 2 and 3.

Microbial screening

Semen cultures revealed aerobic growth in 33% of ejaculates, with potentially pathogenic species in 21% of these specimens (mostly enterococci, group B streptococci, *Escherichia coli* and *Proteus* spp.). Statistically significant was detected between aerobic culture and pus cells count (correlation coefficient =.553.)

Prevalence *of C. trachomatis:* six cases (30%) of the sample from the infertile group gave 241 bp DNA band in agarose gel electrophoresis , in comparison to 5% among the control group (Fig.1),

Prevalence *of of M. genitalium*: Evidence of *M. genitalium* was found in 4 cases (20%) of the infertile group gave 495 bp DNA band compared to 5 % in the control group (Fig.1).

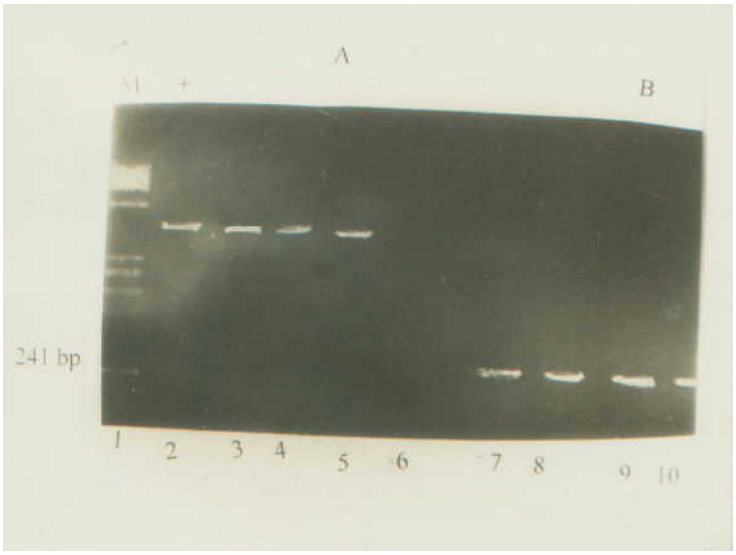

Figure (1): A representing gel stained with ethidium bromide : Lane(1) shows HaeIII-digested phagex174 molecular size marker. Lane (2-5) show the positive cases for the amplified 495-bp fragment from *M. genitalium*. Lane (6) shows :negative control, while lanes (7-10) show the positive cases for chlamydial plasmid containning the amplified chlamydial plasmid at 241 bp

IL-8 determination and result of semen analysis

Positive aerobic cultures were not related to IL-8 concentrations in seminal fluid with correlation coefficient =.096. While IL-8 concentration give significantly correlation with *C.trachomatis* detection (0. 592) . This man had a very high IL-8 concentration in his seminal plasma (139 pg/ml). On the other hand, there was also no correlationship between IL-8 concentration and detection of *M. genitalium* with correlation coefficient

=.318. The association of IL-8 concentrations with results of microscopical ejaculate analysis in fertile and infertile group are shown in Table 2,3

Table(1) Correlation ship between IL8 and semen parameters among the control group

Correlations[a]

			IL_8	SEMEN_C	motility %	PUS_CELL
Kendall's tau_b	IL_8	Correlation Coefficient	1.000	.405	-.045	-.126
		Sig. (2-tailed)	.	.106	.857	.636
		N	10	10	10	10
	SEMEN_C	Correlation Coefficient	.405	1.000	-.068	-.255
		Sig. (2-tailed)	.106	.	.787	.342
		N	10	10	10	10
	motility %	Correlation Coefficient	-.045	-.068	1.000	.459
		Sig. (2-tailed)	.857	.787	.	.087
		N	10	10	10	10
	PUS_CELL	Correlation Coefficient	-.126	-.255	.459	1.000
		Sig. (2-tailed)	.636	.342	.087	.
		N	10	10	10	10
	aerobic	Correlation Coefficient	-.228	.296	-.296	-.443
		Sig. (2-tailed)	.425	.304	.304	.151
		N	10	10	10	10
	PCR_C_T	Correlation Coefficient	.447	.226	-.302	-.296
		Sig. (2-tailed)	.117	.432	.295	.338
		N	10	10	10	10
	PCR_M_G	Correlation Coefficient	-.447	.113	-.188	.085
		Sig. (2-tailed)	.117	.694	.513	.784
		N	10	10	10	10

a. GROUP = fertile men

- Semen_ C: semen count
- PCR _ C_T: PCR for *C .trachomatis*
- PCR _ M_G:PCR for *M. genitalium*

Table(2) Correlation ship between IL8 and semen parameters among the infertile group

Correlations[a]

			IL_8	SEMEN_C	motility %	PUS_CELL
Kendall's tau_b	IL_8	Correlation Coefficient	1.000	-.086	-.142	.252
		Sig. (2-tailed)	.	.603	.395	.137
		N	20	20	20	20
	SEMEN_C	Correlation Coefficient	-.086	1.000	.185	-.067
		Sig. (2-tailed)	.603	.	.267	.692
		N	20	20	20	20
	motility %	Correlation Coefficient	-.142	.185	1.000	-.017
		Sig. (2-tailed)	.395	.267	.	.921
		N	20	20	20	20
	PUS_CELL	Correlation Coefficient	.252	-.067	-.017	1.000
		Sig. (2-tailed)	.137	.692	.921	.
		N	20	20	20	20
	aerobic	Correlation Coefficient	.096	-.213	-.090	.553**
		Sig. (2-tailed)	.621	.270	.647	.006
		N	20	20	20	20
	PCR_C_T	Correlation Coefficient	.592**	-.008	-.114	.292
		Sig. (2-tailed)	.002	.967	.562	.144
		N	20	20	20	20
	PCR_M_G	Correlation Coefficient	.318	.010	.042	.278
		Sig. (2-tailed)	.100	.958	.832	.163
		N	20	20	20	20

**. Correlation is significant at the .01 level (2-tailed).

a. GROUP = infertile men

DISCUSSION

The role of cell-mediated immunity in the aetiopathogenesis of male infertility is far from being defined. The cytochemokine interleukin-8 (IL-8) has a key role in T-cell mediated immune responses [7].

We detect significant difference of IL-8 in seminal fluid between fertile and infertile men, in accordance to **Shimoya et al.,** [16]and **Wu et al.,** [17] .

The correlation between cytokines and infertility explained as cytokines may accumulate and activate leukocytes in the male genital tract, where activated leukocytes produce large amounts of elastase [18] . It has also been shown that polymorphonuclear (PMN) elastase is an inhibitor of sperm motility [10], and that secretory leukocyte protease inhibitor (a potent inhibitor of leukocyte elastase) in seminal plasma reduces the extent of motility inhibition caused by elastase[19] .

Also**, Eggert-Kruse et al.**[8]**,** explained the effect of IL-8 on infertility ,as the role of IL-8 elevations may represent part of a non-specific acute-phase response, or they may be due to specific interactions between viruses (or other stimuli) and the immune system. The subsequent interaction between virus and inflammatory cytokines could lead to a state of (silent) inflammation (possibly with the generation of reactive oxygen species) which could induce suppression of adequate spermatogenesis .

Recently, it is reported by **Celinska et al.** [9] that measuring the level of cytokines, in seminal plasma does not only expand the diagnostic options, but also, through the growing knowledge of immune processes, can give rise to new therapeutic methods of improving the quality of semen and increasing the chance to reproduce .

In the current research ,no significantly correlation was detected between IL-8 concentrations and different semen testing including sperm count ,motility in

accordance to **Koumantakis et al.** [20] and **Zhang et al.** [21]

Furuya et al. [22] reported that certain kinds of cytokine in the seminal plasma might play an important role in improving semen quality **.**

Although , the seminal plasma concentration of IL-8, an important mediator of inflammatory processes and is significantly associated with seminal leukocyte the as reported by **Eggert-Kruse et al.** [8] but we detected that among ,the infertile men, the concentration of IL-8 in seminal fluid was not significantly correlated with leukospermia

One of the theory support our results, was documented by **Leutscher et al.** [23] as in Schistosoma haematobium–endemic the level of cytokines can significant increase due to egg-induced inflammation in the semen, without leukospermia .

Another explanation of increased concentration of cytokines , as an increased production of some pro-inflammatory cytokines has also been reported during immune responses in major depression [24]. Genetic factors also substantially influence the production of cytokines [25].

With regard to other frequently found bacteria of potentially pathogenic nature there was no relationship with IL-8 concentrations. **Eggert-Kruse *et al*.,** [8] reported that in the clinical setting, there is much controversy about the consequences of positive semen cultures in asymptomatic infertile males, this surely depends on the pathogenicity of the species identified in semen. Also cytokine concentrations may more accurately indicate an early phase of infection/inflammation. This is also in agreement with previous studies which did not show an association of seminal bacteria with leukocytes in ejaculates of asymptomatic subfertile patients[26]**.**

The most significant value in this study is the correlation between IL8 and *C.trachomatis* detection by PCR. *C. trachomatis* is very important for subsequent fertility: sexual transmission would have severe sequelae, for example for tubal function in their partners [27]. These microorganisms were detected by high prevalence (30%) by the use of sensitive methods for their detection.

Recently, **Mazzoli et al.,** [28] reported that seminal IL-8 appear to be the best immunologic markers of chronic chlamydial prostatitis status .

It is important to note that the *C. trachomatis*-positive patient in our research had high interleukin concentrations in seminal plasma, indicative of genital tract infection. More research in large groups of males with actual chlamydial infection is needed.

Numerous studies clearly indicate that mycoplasmas are able to modulate the activities of various immune cells and thus trigger the production of a wide variety of inflammatory and anti-inflammatory cytokine[29]**,** *M. genitalium* was selected in this study as it represent 11-35% of men with nongonococcal urethritis, and range from 0-9% among asymptomatic men [30]**.**

In the current research *M. genitalium* detection (20%) was not correlated with IL8 concentration, this findings in the selected asymptomatic group of patients confirmed that no pathological role can be attributed to these bacteria and their presence reflects a silent colonization as reported by **Pannekoek et al.**[11]or the asymptomatic patients showed fewer organisms [31]

Bacterial infection is not the only factor that might lead to an indirect association between leukocytospermia and infertility, alternative aetiologies include abnormal spermatozoa, chemical irritants and environmental factors, as well as viral infections. In most studies concerning microbial findings in semen samples, viruses are not considered, but such knowledge is limited and viruses that might have a significant effect on spermatogenesis have still to be defined. In this respect, interleukins (especially IL-8) are of particular interest [8].

Recently **Khadra et al.,** [32] reported that semen IL-8 levels correlate with subjective symptoms in men and so IL-8 might contribute to the pathophysiology of chronic prostatitis and non specific urethritis and elevated levels might be a useful marker of the condition **,**but in the current study the studied patients are asymptomatic makes our result differ.

In the current research, the assay for cytokine determination were performed in aliquots from the same ejaculates that were used to examine other variables such as standard sperm analysis sperm migration

testing, leukocyte counting and microbial evaluation. This allowed adequate comparison to be made testing was performed under standardized conditions.

In conclusion , IL-8 detected in seminal plasma are associated with the pathogenesis of infertility but not with the pathogenesis of leukocytospermia, and silent genital tract infection in asymptomatic men should depends only on laboratory parameters and not on the level of cytokines detection . IL8 concentration significant correlate with *C.trachomatis* detection .The elevated level of IL-8 among infertile group may be of importance in specific diagnosis and treatment of male infertility.

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مستوى الانترلوكين - ٨ فى بلازما السائل المنوى للرجال العقيمين مع عدوى الجهاز التناسلى الساكنة

نسرين صلاح عمر[١] - سناء محى الدين[١] - يسرى مصطفى [٢]

قسم الميكروبيولوجيا و المناعة الطبية[١] - قسم الامراض الجلدية و التناسلية[٢] - كلية الطب - جامعة المنصورة

هدف هذه الدراسة اثبات وجود الانترلوكين - ٨ و مقارنة مستواه فى بلازما السائل المنوى للرجال العقيمين و اخرون اصحاء و كذلك لدراسة العلاقة بين مستوى الانترلوكين -٨ و عدوى الجهاز التناسلى الساكنة وقد تم اخذ ٢٠ عينة من بلازما السائل المنوى للرجال العقيمين و١٠ اصحاء و تم فحص هذه العينات من حيث اختبارات العقم الاساسية-عد كرات الدم البيضاء –قياس نسبة الانترلوكين -٨ باستخدام اختبار الاليزا

او للكشف عن البكتيريا فى السائل المنوى تم زراعة العينات على مستنبتات هوائية و للكشف عن وجود الكلاميديا تراكوماتس و الميكوبلازما التناسلية استخدم اختبار التفاعل التسلسلى البوليماريزى و قد اظهرت الدراسة و جود علاقة لها دلالة احصائية بين مستوى الانترلوكين - ٨ فى بلازما السائل المنوى بين للرجال العقيمين و الاصحاء

و لكن لا يوجد علاقة مع عدد كرات الدم البيضاء فى بلازما السائل المنوى و ايضا لا يوجد علاقة لها دلالة احصائية بين الانترلوكين - ٨ اختبارات العقم الاساسية مثل حركة الحيوانات اتمنوية فى كلا من المجموعتين المختبرين . كانت نتيجة الزرع على المستنبتات الهوائية ظهور بكتيرية هوائية فى ٣٣% من الحالات ومنهم نسبة ١٢% بكتيرية ممرضة و قد وجدت علاقة لها دلالة احصائية بين البكتيريا الهوائية و عدد كرات الدم البيضاء فى بلازما السائل المنوى. و قد ظهرت من نتائج التفاعل التسلسلى البوليماريزى لعينات بلازما السائل المنوى للرجال العقيمين ان نسبة ٣٠% منها توجد بها الكلاميديا تراكوماتس و الميكوبلازما التناسلية بنسبة ٢٠% اما فى الاصحاء فكانت النسب ٥% لكل من الميكروبين و قد استنتجنا ايضا ان المزارع الهوائية الايجابية لاتوجد لها علاقة ذات دلالة احصائية مع تركيز الانترلوكين - ٨ ووجدت فقط هذه العلاقة مع وجود الكلاميديا تراكوماتس .

الخلاصة انه لايمكن الاعتماد على مستوى الانترلوكين – ٨ فى عينات بلازما السائل المنوى للرجال العقيمين لِتشخيص عدوى الجهاز التناسلى الساكنة و لكنه له علاقة بالعقم ويمكن استخدامه لتشخيص العقم فى الرجال.