

## Correlation of Seminal Fluid -Immunologic Inflammatory Markers with Cotinine Concentration in Smokers -Unexplained Infertile- Males

ارتباط الواسمات الالتهابية المناعية بتركيز الكوتينين في السائل المنوي عند الرجال المدخنين المصابين بالعقم الغير مفسر

Dr. Nael Hussein Ali \*

### الخلاصة

**الهدف:** لتبيان الارتباط التسببي لتدخين السجائر (مقاس بتركيز مستوى الكوتينين في بلازما المنوي) بالواسمات الالتهابية المناعية في بلازما السائل المنوي (اليستيز الخلايا المحببة متعددة النوى والساييتوكاينات ما قبل الالتهاب و الخلايا البيض في المنوي) في السائل المنوي للرجال المصابين بالعقم الغير مفسر. **المنهجية:** مائتان وستة وثلاثون رجلا مصابا بالعقم الغير مفسر منهم مائة و اربعون مدخنين وستة وتسعون غير مدخنين . تسعون رجلا خصيبا يبدو بصحة جيدة وغير مدخن اعتبروا المجموعة الضابطة. تم قياس الواسمات الالتهابية والمناعية في السائل المنوي (اليستيز الخلايا المحببة متعددة النوى وأنترلوكين-8 وأنترلوكين-6 و الخلايا البيض في المنوي) و مستوى تركيز الكوتينين في السائل المنوي (باستخدام الأليزا) عند المشتركين كافة والبالغ عددهم ثلاثمائة ومائتان وستة عينة.

**النتائج:** الفروقات بين الواسمات الالتهابية المناعية وتركيز الكوتينين كانت فروقات معنوية عند مجاميع الدراسة الثلاثة والارتباط ذات معنوية احصائية بمستوى (اقل من 5%).

**الاستنتاج:** التأثير السلبي للكوتينين على حركة وشكل الحيامن وارتباط ذلك بشكل معنوي احصائيا بالواسمات المقاسة يدل دلالة قوية على وجود رد فعل مناعي في القناة التناسلية للرجال المدخنين. مما ادى ذلك الى التهابات المجاري التناسلية الغير ظاهر (دون السريري) الامر الذي كان سببا في حالة العقم الغير مفسر لدى هؤلاء الرجال. **التوصيات:** يجب نصح الرجال العقيمين بالابتعاد عن دخان السجائر سلبيا او ايجابيا.

### ABSTRACT

**Objective:** To determine the causing correlation of the cigarettes smoking (measured by cotinine level in the seminal plasma) on the immunologic inflammatory markers (PMN-elastase, pro-inflammatory cytokines, and leukocytospermia) in seminal fluid (SF) of unexplained infertile men.

**Materials and Methods:** Tow hundred-thirty six unexplained infertile men, one hundred fourteen smoking and ninety six non-smokers were included in the study. They were being found without evident cause for infertility after initial workups. Other ninety fertile healthy non-smokers men were enrolled in the study as a control group. Seminal immunologic inflammatory markers (PMN-elastase, pro-inflammatory cytokines IL-8 and IL-6, and leukocytospermia) were measured in seminal plasma (SP) of all participants. Cotinine level in SP was measured as well (by using ELISA), and correlated with inflammatory markers in all three hundred- twenty six specimens.

**Results:** The differences of seminal plasma inflammatory markers and cotinine level were significant between the three groups of the study, and significantly correlated ( $P<0.05$ ) with inflammatory markers.

**Conclusion:** The resulted substantial negative impact on sperm motility and morphology, and the correlation of cotinine level with immunologic inflammatory markers in SP, strongly indicate the presence of immunologic reaction in genital tract of the smokers. That may lead to silent genital tract inflammation. Which is, may be, the main cause for their unexplained infertility.

**Recommendation:** It would be sensible to advise infertile men to abstain from smoking cigarettes.

**Keywords:** Cigarette smoking; cotinine; infertility; Silent male genital tract inflammation; Seminal plasma inflammatory markers.

Assist. Prof. , M.Sc. Medical Microbiology , Ph.D., Clinical Immunology- Dean College of Nursing/University of Basrah , Basrah-IRAQ  
[nhali66@yahoo.com](mailto:nhali66@yahoo.com)

## INTRODUCTION

One third of the world's population continues to consume cigarettes on regular basis, despite worldwide anti-smoking campaigns <sup>(1)</sup>. A causal relationship between cigarettes smoking and impaired reproductive function is highly suspected because smokers inhale a host of toxins <sup>(2)</sup>. Tobacco combustion yields about 4000 compounds; the smoke can be divided into a gaseous phase and a particulate phase. The principal harmful components of the gaseous phase are carbon monoxide, nitrogen oxide, ammonia and volatile hydrocarbons <sup>(3)</sup>. The main components of the particulate phase are nicotine and cadmium. Nicotine is quickly absorbed through the respiratory tract, mouth mucosa and skin <sup>(4)</sup>.

About 80% to 90% of nicotine is metabolized by body organs; mainly by the liver, but also by the kidneys and lungs. Its major metabolite is cotinine <sup>(5, 6)</sup>. Nicotine and its metabolites have been detected in serum, urine <sup>(7)</sup>, and recently they have been found at significant levels in smokers' seminal plasma and in subjects exposed to environmental tobacco smoke <sup>(8)</sup>.

Given that cigarette smoke known to be mutagens, aneugens, or carcinogens <sup>(1, 9-11)</sup>. Smoking cigarettes was claimed by many authorities <sup>(12-15)</sup> to causing alteration in sperm quality such as semen volume, sperm concentration, motility and morphology. Others <sup>(16)</sup> did not find any adverse affect on sperm parameters in a population of Swedish infertile men. The effect of nicotine on male fertility remains controversial and several mechanisms have been suggested as; being responsible for the induction of abnormalities <sup>(17)</sup>; inhibition of creatine kinase activity for normal sperm energy <sup>(18)</sup>; nicotine and its soluble metabolite (cotinine) could be pass through the blood-testis barrier <sup>(7)</sup>; and endocrine effect of smoking on fertility has also been suggested <sup>(19)</sup>. Furthermore, smoking has been associated with an increase in oxidative stress within the seminal environmental leaving sperm vulnerable to oxidative damage <sup>(20, 21)</sup>. Damage at the DNA level and chromosomal aneuploidy has been resulted by many studies <sup>(22- 24)</sup>. More recently, apoptotic markers in semen of smoking infertile men have been resulted in Egypt by El-Melegy *et al.*, <sup>(25)</sup>. The relationship between immunologic factors, including cytokines, and human reproduction represents a growing area of investigation because of their involvement in different aspects of reproductive physiology and fertility regulation <sup>(26)</sup>. The increased levels of these factors in semen from men with genital infections suggest their role in immune defense of the male genital tract <sup>(27, 28)</sup>.

Pro-inflammatory cytokines increasing with other inflammatory markers in seminal fluid in response to external stimuli or inflicted injury, modulate inflammatory reactions lead to leukocyte migration to the site of inflammation where they mobilize the host defense system <sup>(29)</sup>, a matter which can affect spermatozoa.

Interleukin-6 (IL-6) is one of proinflammatory cytokines, is produced by activated Th2 cells and other somatic cells, and promotes the replication of B-cells and production of immunoglobulin. IL-6 does not induce the production of other cytokines; however, it does act synergistically with IL-1 $\alpha$  to promote T-cell activation <sup>(28)</sup>. IL-8 is a chemokine produced by macrophages and other somatic cells, and acts as a chemoattractant for neutrophils and T cells. Because of their clear roles in inflammation, these cytokines are good candidates to be a marker for inflammation and/or infections <sup>(30)</sup>.

Available data of the possible detrimental effects of cigarettes smoking on male reproductive performance and specifically on immunologic inflammatory markers in Seminal Fluid (SF) is quiet conclusive and is of great interest.

The aim of this study is determine the causing effect of the cigarettes smoking (measured by cotinine level in the seminal plasma) on the immunologic inflammatory markers (PMN-elastase, pro-inflammatory cytokines, and leukocytospermia) in SF of unexplained infertile men.

## **MATERIALS AND METHODS**

**Study groups:** This case-control study was conducted at the Basrah Infertility Center between July, 2014 and October, 2015. One hundred fourteen smoking men with age of 22-42 years, and other ninety six non-smokers with age of 24-44 years were included in the study. Both groups were suffered from primary unexplained infertility, when they failed to conceived over 12 months of marriage. They were married from 1-10 years, which was a period of their infertility. They were being found without evident cause for infertility after initial workups, including:

1. Semen analysis ranged from oligo - to normozospermia;
2. No previous urogenital surgery nor any hydrocele or varicocele grades; undescended testis or its corrective surgery; Vasectomy-reversal surgery; no overt signs of urethritis or prostatitis.
3. Not infected with chronic illness like tuberculosis, malaria, HIV, etc. or pyospermia, haemospermia or chronic urinary tract infection.
4. Normal hormonal assay (Testosterone, prolactine, FSH, and LH).
5. Not exposed to gonadotoxines (chemotherapy, radiotherapy, or pesticides).
6. Their spouses were apparently fertile women.

The inclusion criteria; for smokers group, smokes cigarettes on a regular basis (at least 20 cigarettes/day for at least one year before enrollment in the study); for non-smokers, never smoked before.

Other ninety fertile healthy non-smokers men with age of 25-43 years were enrolled in the study as a control group, they have at least one child within last two years without assisted reproductive techniques.

Criteria of exclusion were:

1. Men with known cause for their infertility.
2. Those suffering from azoospermia or secondary infertility.
3. Patients with a history of recreational drug use or alcohol consumption.
4. Those with history of prolonged medication intake, herbal preparations or tonics.
5. Occupational exposure to chemicals or excessive heat.
6. Over 45 years old.

### **Seminal Fluid processing:**

A total of three hundred-twenty six semen samples were collected. Each specimen was collected by masturbation in sterile container following 2-5 days of sexual abstinence. The specimens were allowed to liquefy for 20-30 minutes at room temperature. An aliquot of each semen samples was subjected to routine semen analysis in accordance with the guidelines published by WHO <sup>(32)</sup>. Seminal fluid parameters included; semen volume (ml), PH, motility graded (A, B, C, D), morphology, sperm count ( $10^6/\text{ml}$ ) and leukocytes count.

### **Pro-inflammatory cytokines detection in Seminal Plasma (SP):**

Each semen sample was, then, centrifuged at 400xg for 10 minutes at room temperature to obtain the seminal plasma (SP) fraction. Phenyl methyl sulphonyl fluoride (PMSF), a protease inhibitor, was then added to the SP. The PMSF- treated SP fractions frozen at  $-20^{\circ}\text{C}$  until use.

Immediately prior to cytokines determination, frozen SP specimens were thawed at room then stored in microfuge tubes. Quantization of the proinflammatory cytokines was done using ELISA kits; IL-6 and IL-8 (Quantikine®; R & D Systems, Minneapolis MN, USA). Briefly, these were ELISA with a quantitative sandwich technique using microtitre plates which were pre-coated with monoclonal antibodies specific for each cytokine. After reaction, the resulting color reaction was proportional to the amount of the considered cytokine and was measured spectrophotometer (Backman Coulter, Germany) at 450 or 500 nm. Testing was performed strictly according to the manufacturer's instructions.

#### **PMN-elastase assay:**

By using ELISA kit (PMN-elastase ELISA, Merck Darmstadt, Federal Republic of Germany) in cell free SP PMN-elastase was determined. Any participant showed PMN-elastase concentration >250 ng/ml were considered as having a genital tract inflammation (silent inflammation).

#### **Cotinine level measuring:**

Cotinine due to its longer half life has been used as a reliable marker for exposure to tobacco smoke. The Cotinine Direct ELISA Kit (Diagnostic Products Corp, Los Angeles, CA, USA) is a specific and sensitive *in vitro* test to detect the presence of cotinine in seminal plasma. Competitive binding to antibody of enzyme labeled antigen and unlabeled antigen, in proportion to their concentration in the reaction mixture. A 10 µl aliquot of a diluted unknown specimen is incubated with a 100 µl dilution of enzyme (Horseradish peroxidase) labeled cotinine derivative in micro-plate wells, coated with fixed amounts of oriented high affinity purified polyclonal antibody. The wells are washed thoroughly and a chromogenic substrate added. The color produced is stopped using a dilute acid stop solution and the wells read at 450 nm. The intensity of the color developed is inversely proportional to the concentration of cotinine in the sample. The technique is sensitive to 1 ng/ml.

#### **Statistical analysis:**

All parameters expressed as Mean  $\pm$  SD using and the analysis of data was conducted by using the available software of Statistical Package for Social Sciences (SPSS) version 21 employing analysis of variance (ANOVA) as well as Pearson correlation statistics. The correlation is significant at the 0.05 level (2-tailed).

## RESULTS:

**Table (1) Descriptive comparison between smokers, non-smokers infertile, and fertile men in SF parameters**

SF parameters	Infertile smokers (N=140 )	Infertile non-smokers (N= 96 )	Fertile(control group) (N= 90 )	P value
	Mean $\pm$ SD (range)	Mean $\pm$ SD (range)	Mean $\pm$ SD (range)	
Volume /ml	2.7 $\pm$ 0.5 (2.5 - 3.1)	2.9 $\pm$ 0.5 (2 - 3.8)	2.7 $\pm$ 0.9 (2 - 3.5)	0.593
PH	7.7 $\pm$ 0.2 (7.5 – 8.0)	7.8 $\pm$ 0.2 (7.5 – 8.2)	8.0 $\pm$ 0.5 (7.2 – 8.1)	0.041
Count(10 <sup>6</sup> /ml)	45.1 $\pm$ 3.0 (32 - 52)	43 $\pm$ 3.7 (37 - 50)	44.4 $\pm$ 3.7 (35 - 53)	0.575
Motility A Grades %	20.5 $\pm$ 15.8 (0-5)	18.1 $\pm$ 11.4 (0-5)	62.5 $\pm$ 11.2 (20-55)	0.001
B	32.5 $\pm$ 13.6 (0-15)	10.5 $\pm$ 4.6 (0-10)	10.75 $\pm$ 4.67 (10-20)	0.001
C	38.2 $\pm$ 10.1 (0-30)	45.2 $\pm$ 14.1 (0-10)	32.5 $\pm$ 4.13 (0-20)	0.132
D	42.6 $\pm$ 28.1 (0-60)	58.5 $\pm$ 36.7 (0-75)	20.0 $\pm$ 6.7 (0-5)	0.010
Morphology: Normal %	23.7 $\pm$ 17.8 (15- 25)	30.7 $\pm$ 10.6 (22 - 40)	60.6 $\pm$ 22.7 (33 -70)	0.017
Abnormal %	76.5 $\pm$ 10.9 (40 -75)	70.5 $\pm$ 13.6 (45 - 60)	40.5 $\pm$ 80.9 (20 - 30)	0.176

**P < 0.05 statistically significant**

This table shows the differences between all three groups are significance (P < 0.05) in the PH, motility grades, and morphology SF parameters.

**Table (2): Descriptive comparison between smokers, non-smokers infertile, and fertile men in SP inflammatory markers and SP cotinine level**

SP Inflammatory markers	Infertile smokers (N=140 ) Mean $\pm$ SD (range)	Infertile non-smokers (N= 96 ) Mean $\pm$ SD (range)	Fertile(control group) (N= 90 ) Mean $\pm$ SD (range)	P value
IL-6 (pg/ml)	9.4 $\pm$ 7.7 (0.9-62.3)	7.5 $\pm$ 6.8 (6.6-76.8)	6.2 $\pm$ 4.4 (0.5-56.2)	0.554
IL-8 (pg/ml)	668.5 $\pm$ 170.3 (434.6-866.7)	334.7 $\pm$ 199.7 (235.9-409.5)	208.9 $\pm$ 65.8 (101.9-309.3)	0.002
Leukocytospermia ( $> 10^6$ /ml)	87 (62%)	34 (35%)	32 (35%)	0.017
PMN-elastase ( $>250$ ng/ml)	89 (63%)	32 (33%)	29 (32%)	0.019
Cotinine level ( $\mu$ g/dl)	1354.23 $\pm$ 65.19 (200.7-1401.8)	176.45 $\pm$ 22.79 (65.6-308.6)	164.30 $\pm$ 22.68 (52.4-185.8)	0.001

**P < 0.05 statistically significant.**

The range and mean of seminal plasma inflammatory markers (IL-6, IL-8, Leukocytospermia and PMN-elastase) and cotinine level are shown in the table (2). Their differences always are significant (P < 0.05) between the three groups of the study.

**Table (3) correlation values of SP Cotinine with immunologic inflammatory markers in SP of the three groups.**

SP Inflammatory markers	Infertile smokers	Infertile non-smokers	Fertile(control group)
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<b>IL-6 (pg/ml)</b>	<b>0.306**</b>	<b>0.212</b>	<b>0.112</b>
<b>IL-8 (pg/ml)</b>	<b>0.232**</b>	<b>0.143**</b>	<b>0.263</b>
Leukocytospermia ( <b>&gt; 10<sup>6</sup>/ml</b> )	<b>0.535**</b>	<b>0.470*</b>	<b>0.128</b>
<b>PMN-elastase</b> ( <b>&gt;250 ng/ml</b> )	<b>0.411**</b>	<b>0.349*</b>	<b>0.445*</b>

• Correlation is statistically significant at level of < 0.05

•• Correlation is statistically significant at level of < 0.001

The SP cotinine level is significantly correlated ( $P < 0.05$ ) with all inflammatory markers in the infertile smokers group, and significantly correlated ( $P < 0.05$ ) with inflammatory markers except IL-6 in the infertile non smokers group, while it is non-correlated significantly with inflammatory markers of fertile non smokers group except with PMN-elastase (Table-3).

## DISCUSSION

The World Health Organization estimates that nearly 1 billion men and 250 million women worldwide smoke a combined total of more than 15 billion cigarettes daily. To our knowledge, this is the first study to date providing information on correlation between cigarettes smoking measured by cotinine level and immunologic inflammatory markers in SF.

Cotinine is the most commonly used biomarker of exposure to cigarette smoke, because it has a longer half-life (average, 18 to 20 hours) than nicotine does (average, 2 to 3 hours) <sup>(31)</sup>. However, single-nucleotide polymorphisms within the gene encoding CYP2A6 have been noted and can affect a person's ability to metabolize nicotine to cotinine <sup>(32)</sup>.

To overcome the potential source of any bias, the participants of the present study were selected within strict criterion of inclusion. The unexplained infertile smoking men group was compared with other two groups of non smokers one of them are unexplained infertile men as well. Furthermore, in infertility cases, it must be borne in mind that a part from the alterations in classical sperm parameters, tobacco compound may affect sperm quality in other ways. Biochemical changes that may reduce sperm quality have been documented in SF of smokers and genotoxicity of tobacco smoke is indisputable.

In correspondence with other studies <sup>(8,14)</sup>, the present study resulted a substantial negative impact on sperm motility and morphology. On the contrary, some researchers reported different results. Adelusi *et al*, <sup>(33)</sup> found that smoking significantly increased sperm motility among infertile men, with no apparent mechanism. Trummer *et al*, <sup>(34)</sup> showed that smoking did not affect conventional semen variables, but significantly increased round cells and leukocytes. Ozgur *et al*, <sup>(35)</sup> showed that heavy smokers have a higher percentage of rapidly progressive spermatozoa than light smokers. Hassa *et al*, <sup>(31)</sup> showed that cigarette smoking was not correlated with sperm concentration, total motile sperm count and sperm morphology.

The effect of nicotine consumption on male reproduction function remains under discussion. Several studies were admonished by some authorities <sup>(2, 19)</sup> for the few participants and lack of smoking dose data leading to this dissociation. Experimental study have indicated that in male rats exposed to smoking, serum level of nicotine and cotinine were increased which adversely affected spermatogenesis and sperm fertilizing potential <sup>(28)</sup>. Other rats exposed to cigarette smoke for 7-10 weeks, Kapawa and his colleagues <sup>(17)</sup> found a secretory deficiency of Leydig and Sertoli cells, leading to impaired epididymal sperm maturation and a diminished capacity of spermatozoa to penetrate oocyst.



The presence of immunologic inflammatory markers in conjunction with the absence of any clinical features may indicate that the smokers may have silent genital inflammation and reflect changes in local immune mechanisms as a part of the general immunorections <sup>(36)</sup> whether the increase of immunologic inflammation markers (because of smoking) is local or systemic need for further investigation.

The level of cotinine was inversely proportional to semen density and motility. Controlling for potential confounding variables, such as seasonal variation for semen collection, age, race, or associated caffeine consumption had no effect on these correlations, emphasizing the pure effect of smoking on male fertility contemporary.

The results of present study showed that the seminal plasma concentration of IL-8, an important mediator of inflammatory processes, is significantly associated with seminal leukocytes and with some clinically relevant parameters of semen quality. Interleukin-8 is a potent neutrophils chemotactic and activating factor. It is involved in angiogenesis and adhesion processes, and enhances the adherence of neutrophils to endothelial cells and subendothelial matrix proteins. It exerts its biological activities by binding to specific cell surface receptors <sup>(29)</sup>. Therefore, granulocytes are the main constituents of the white blood cells population in semen. One of the main changes during the inflammatory process is the discharge by these polymorphonuclear granulocytes of large amounts of proteases such as elastase. As the evolution of elastase in seminal plasma is of clinical importance in detecting inflammation, immunologic measurement of PMN-elastase was considered and proved as a reliable marker of silent tract inflammation <sup>(36)</sup>, and the absence of leukocytes in semen does not exclude the possibility of inflammation and/or infection reactions in the male genital tract<sup>(37)</sup>. Therefore, in the present study PMN-elastase was used beside leukocytospermia as an immunological inflammatory marker.

The exact mechanism of increased immunologic inflammatory markers in SF of infertile smokers is not clear and warrants for further research. Several speculations may explain this situation. Firstly, the cotinine may induce inflammatory reaction in the male genital tract lead to release of chemical mediators of inflammation involving IL-6 and IL-8 <sup>(30)</sup>. These cytokines can recruit and activate leukocytes especially polymorph nuclear leukocytes, which are the cause for increasing of PMN-elastase in SF. Secondly, the cotinine (as a toxic metabolite from nicotine) may impair spermatogenesis, resulting in the production of defective spermatozoa, a matter which induce leukocytes to infiltrate the male reproductive tract to eliminate defective spermatozoa by phagocytosis, and so, other immunologic inflammatory mediators may elevate. Another speculation is that, the cigarette smoke itself contains high levels of reactive oxygen species such as superoxide anion, hydrogen peroxide, and hydroxyl radicals. Spermatozoa are susceptible to damage by these free radicals. Because the spermatozoa cytoplasm contains low concentrations of scavenging enzymes, their plasma membrane contain large quantities of polyunsaturated fatty acids.

The limitation in the present study should be mentioned, smoking data were obtained from participants as the number of cigarettes smoked per day and duration of smoking in years. That may be not validated to test in serum or salivary cotinine levels. Also I was unable to examine the relationship of cotinine with semen parameters in a dose-dependent because of the subjective nature of the smoking history. Other limitation is the shortage of references analogues with this study.

## CONCLUSION



A conclusion can be reached as to potential negative association between cotinine (as an oxide metabolite of nicotine) in infertile men. Smoking cigarettes may be considered as a causative factor for infertility.

## RECOMMENDATIONS

1. Cessation of smoking cigarettes to avoid decreased fertility.
2. Negative smoking effect on fertility and other types of smoking needs for further clarification.

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