

Tumour Necrosis Factor and Sperm Nuclear DNA Integrity in Semen of Infertile Males

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Abstract: Background: TNF is a proinflammatory cytokine that has recently been blamed to cause male infertility. Pathologically high levels Sperm DNA fragmentation has emerged as a potential causative factor of reproductive failure as well as failure to conceive with intra-cytoplasmic sperm injection (iczi). Anti-sperm antibodies (ASA), varicocele (VC), and genital Infection with or without inflammation & leukocytospermia can cause male infertility directly or indirectly through pathologically increasing TNF. **Aim of the work:** This study aims to evaluate seminal levels of TNF- α in correlation with sperm parameters and sperm nuclear DNA integrity in infertile males. **Methods:** A Total of 80 male subjects with ages ranging from 25-50 years were included in this study. 40 infertile males compared with 40 healthy fertile males. Patient groups were subdivided into group (A) Varicocele, group (B) leukocytospermia, group (C) genital infection, and group (D) antisperm antibodies. All patients groups were subjected to the following. Seminal fluid TNF- α , COMET assay for sperm nuclear DNA fragmentation, seminal ASA. Lastly seminal analysis to detect sperm parameters, and seminal culture to detect genital infection. **Results:** TNF- α was increased in all patient groups and correlate significantly with defaults in sperm parameters and sperm nuclear DNA fragmentation. TNF- α were significantly increased and correlated with Varicocele grades genital infection and leukocytospermia. Although TNF- α was significantly increased in patients with antisperm antibody but not correlated. **Discussion:** TNF has direct role in infertility either through caspase activation and/or increasing both reactive oxygen species (ROS) and nitric oxide (NO).

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1. Introduction

Infertility is a common problem affecting males of various age and race. A study by Mascarenhas et al., 2012 have shown that the percentage of male infertility in Egypt is about 37.47 % of married males of age groups ranging 25-54 years.

Tumor necrosis factors (TNF- α) refer to a diverse cellular immune mediators group of non-antibody proteins known as cytokines and has been suspected to be a hazard to male fertility. Other cytokine members are interferons, interleukins, lymphokines and chemokines. (1).

TNF- α may pathologically increase by infection and inflammatory conditions of male accessory sex glands (e.g., orchitis, epididymitis, prostatitis and prostatic-vesiculitis) as well as inflammation of the testis, which can occur due to mumps, gonorrhoea and chlamydia (2). Also ischemia-reperfusion injury (varicocele and testicular torsion) and trauma may increase TNF- α (3).

TNF- α is a kind of important cell factor that shows a wide variety of biological actions which may interfere with reproductive functions, as induction of

immune response, chemotactic activity on neutrophils (4) and sperm survival in the female genital tract by aiding, along with the help of other seminal immune modulators, in neutralizing the immune response against spermatozoa in female genital tract. Therefore, TNF- α aids in sperm capacitation which is the final process of maturation of spermatozoa that occurs in female genital tract before fertilizing the oocyte (5).

Male fertility can be affected by proapoptotic effect of TNF- α by recruiting adaptor proteins that activates cysteinyl aspartate-specific proteinases (caspase) which proteolytically degrade a host of intracellular proteins. Also by enhancing ROS (reactive oxygen species) intracellular production that directly causes oxidative stress which damages intracellular proteins and induces mitochondrial cristae reorganization to release caspase activator 'cytochrome C'. Both pathways lead to sperm nuclear / mitochondrial double or single stranded DNA breaks (fragmentation) (6).

Pro-inflammatory cytokines appear to be a natural component of seminal plasma, together with a broad array of immunologic factors, and they are

involved in the physiological function of the male gonad, also acting in the fertilization process. Various cells in the male genitourinary tract release Cytokines and their production increases in response to foreign antigens, pathogens and chronic inflammations.(7)

TNF- α is a proinflammatory cytokine implicated in many physiological and pathological reactions including cell death, cell survival, immune response, and inflammation. TNF- α is a kind of important cell factor that shows a wide variety of biological actions which may interfere with reproductive functions, as induction of immune response, chemotactic activity on neutrophils and sperm survival in the female genital tract by aiding, along with the help of other seminal immune modulators, in neutralizing the immune response against spermatozoa in female genital tract. Therefore TNF- α aids in sperm capacitation, which is the final process of maturation of spermatozoa that occurs in female genital tract before fertilizing the oocyte.(8)

The aim of this study is to evaluate the role of pathological Tumour necrosis factor- α (TNF- α), levels on sperm parameters including sperm DNA fragmentation as a cofactor to male infertility.

2. Patients and Methods:-

A Total of 80 male subjects with ages ranging from 25-50 years were included in this study. 40 infertile males were recruited from the infertility hospital clinics of al-Azhar university. Equally a further 40 healthy fertile males participated as controls.

The subjects were divided into 2 main groups:

Group (I) 40 infertile males, subdivided into 4 subgroups according to clinical and laboratory investigations, revealing oligoathenozoospermia:-

- Subgroup (A) patients with varicocele (7).
- Subgroup (B) patients with leukocytospermia (12).
- Subgroup (C) patients with genital infection (14).
- Subgroup (D) patients with anti-sperm antibodies (7).

Group (II) 40 fertile healthy males as control. ☐

• **Ethical Consideration:** ☐ All participants (patients and controls) in this study were informed about the study process and their informed written consent was taken prior investigations.

Inclusion Criteria:

Infertile males with one or more of the following:-

1. Oligoathenozoospermia.
2. Leukocytospermia / pyospermia.
3. Antisperm antibodies.
4. Varicocele.

Exclusion Criteria:

Patients with:-

1. Azoospermia, Immunocompromised state (eg. Diabetes melitis, uremia, malignancy).
2. Corticosteroids and immunosuppressive treatments.
3. Current or previous treatment with anti-TNF therapy.
4. Chronic heavy smokers.
5. Polluted occupation.

All subjects were submitted for the following:

1. Genitalia examination, scrotal sonar: to detect varicocele.
2. Semen analysis: to assess sperm parameters, according to WHO, 2010.
3. Semen Culture (aerobic & anaerobic): to detect micro- organisms causing genital infection and Assessment of Chlamydia and Mycoplasma infection: by selective culture media.
4. Assessment of anti-sperm antibodies: by ELISA®.
5. Assessment of TNF- α : by ELISA®.
6. Assessment of Sperm Nuclear DNA integrity: by COMET assay®.

I) Semen collection and analysis

1. After a period of 4-7 days' sexual abstinence, semen samples were produced by masturbation, and collected into a sterile wide-mouthed plastic specimen container.

2. After liquefaction at 37°C for 30 minutes, standard comprehensive semen analysis and evaluation of semen parameters were performed according to WHO criteria (2010).

3. Pus cells were identified visually. A concentration over 5/HPF was considered out of range and identified as pyospermia.

4. Leukocytes were identified by peroxidase staining, a concentration over 1 x 10⁶ was identified as leukocytospermia.

II) Semen Culture

Bacteriological analysis was performed on all semen samples in a solid-phase using a quantitative method of assessment. 20 μ l of Semen was seeded on MacConky and blood agar plates in aerobic and anaerobic conditions.

Patients showing quantitative bacteriological culture greater than 10³ pathogenic bacteria or greater than 10⁴ non- pathogenic bacteria in seminal plasma are considered as positive for genital infection.

The most frequently isolated microorganisms in semen were streptococci (eg. St. fecalis, St. haemolyticus), staphylococci (eg. S. aureus AND Staph epidedemus), E. coli in addition to some anaerobic bacteroids, mycoplasma and chlamydia trachomatis.

III) Isolation and separation of sperms from seminal plasma

After semen analysis, all semen samples from participants were immediately centrifuged at 1,500 rpm in the centrifugation chamber for 10 minutes. The separated sperms were immediately analyzed for DNA fragmentation while the remaining seminal plasma was thawed for later assessment of antisperm antibodies and TNF level.

IV) Assessment of ASA

Seminal plasma samples had been stored frozen at -20°C until use. The samples were tested for ASA by ELISA

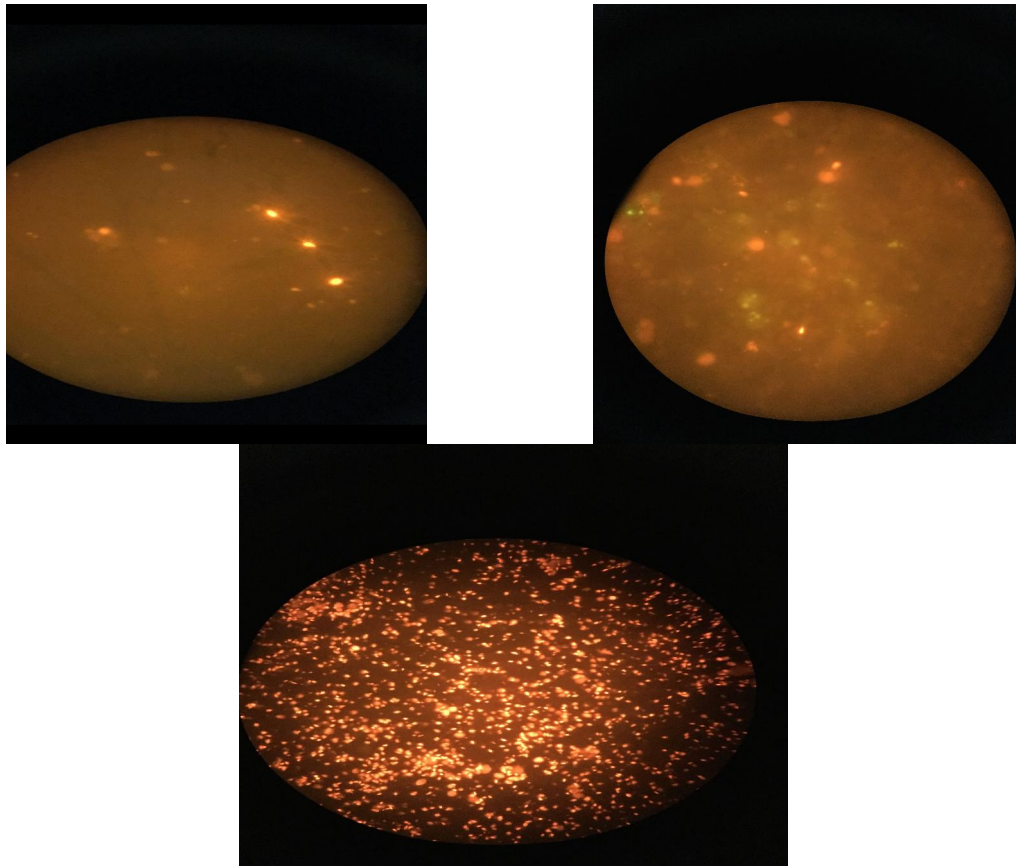
V) Assessment of TNF- α in Seminal Plasma by ELISA

The assay was done by a kit, (Avi-Bion Human TNF- α).

VI) Assessment of Sperm DNA Fragmentation by

COMET assay,

Principle: The COMET assay microscopically detects DNA damage at the level of a single cell, with the aid of fluorescent DNA binding dye (Ethidium bromide (EtBr), the dye measures sperm nuclear DNA fragmentation through binding to the double-stranded nucleic acids as an intercalating dye, so testing the survival of the DNA supercoils for the possibility of free rotation/breaks by causing negative DNA supercoiling upon its addition, the loops expanded out from the nucleoid core would form a “comet”. Sperm DNA damage in this study was quantified by measuring sperm head fluorescent intensity and presence of comet tail.



Technique for Fluorescent microscopy study using comet assay

1. Ethidium Bromide preparation

Staining solution was prepared by adding w/v 1 mg / letter 1% EtBr powder in distilled water to a mixture of BPS

2. Sperm staining with EtBr

- The sperms collected where stained with a 5 μml drop titer of working solution of immune-fluorescent nuclear DNA binding dye EtBr.
- Sperms were washed by BPS following

proper staining.

- Upon addition of ethidium bromide to sperms, a 5 minutes time was given to ensure proper DNA staining, one drop was placed on a slide then examined under the fluorescent microscope at X400 magnification.

- The slides were prepared on the same day with the fluorescent microscope using a 490-nm excitation filter and a 530-nm barrier filter.

- At least 100 cells were counted so that the estimate of the number of sperms with reduced sperm head fluorescent intensity and comet tail presence is accurate.
- The percentage of spermatozoa with fragmented DNA was assessed through observing the sperm head fluorescent intensity. Increased sperm DNA fragmentation was expressed by decreased sperm head fluorescent intensity and ‘comet’ tail presence. 100 comets were visually scored and given a percentage % score to evaluate Sperm fluorescent fragmentation Index. Each comet assigned a grade of 0-4, according to its class.
- Sperms with comet value of 0 were considered normal, while sperms with value ranging from 1-4 were considered abnormally damaged. (9)
- A single observer interpreted the fluorescent sperm head intensity to rule out inter-technician variability.
- The percentage of sperms with damaged DNA was expressed in terms of DNA fragmentation index (DFT%). (10)

DNA Fragmentation Index (DFI%)

- <15% DFI = excellent to good sperm DNA integrity .
- > 15 to < 25% DFI = good to fair sperm DNA integrity.
- > 25 to < 50% DFI = fair to poor sperm DNA integrity.
- > 50% DFI = very poor sperm DNA integrity.

Statistical analysis

Data was analyzed using SPSS V16 (IBM Corp., Armonk, NY, USA). Mean values were calculated for all continuous findings of the values, along with their standard deviations. In order to study associations between two continuous variables, a Pearson’s correlation coefficient was calculated. To study differences in mean findings of related continuous interval groups, an unpaired Student’s t-test or a one-way analysis of variance (ANOVA) was performed when relevant. Differences were considered to be of

statistical significance at $P < 0.05$.

3. Results:-

This study included two groups, patient group and control group each of them was subjected to the same protocol of the study and included 40 patients.

The age range of the patient group was (24-48) years with a mean of (30.90±5.35) and the age range of the control group was (25- 48) years with a mean of (31.85±5.70).

Table 1 shows the mean TNF level of both patients and control groups that appear to be higher in the patient group with highly statistically significant difference between the two groups.

Table 3 shows the mean of the total sperm count per ml of both patients and control groups that was lower in patient group than the control group with statistically significant difference.

Table 4 shows the mean percentage of the progressive motility of sperms of both patients and control groups that were lower in patient group than in control group with highly statistically significant difference.

Table 5 shows the mean percentage of DNA fragmentation in patients group was higher than control group with statistically significant difference.

Table 6 shows the mean leukocytes count per ML in both patients and control groups with evident leukocytospermia in patient group with highly statistically significant difference.

Table 7 shows the varicocele grades in both groups with the higher grades appear more in patients group with statistically significant difference.

Table 8 shows no statistically relation between antisperm antibodies with TNF (pg/ml) in patients group.

Table 9 shows Positive correlation and significant between TNF and Sperm DNA. Fragmentation and leukocytes, while a significant negative correlation and significant between TNF and T. Number, Progressive Motility and varicocele grade.

Table (1): Comparison between patients and control groups according to TNF level (pg/ml).

TNF pg/ml	Patients	Control	t-test	p-value
Mean±SD	133.63±73.39	6.87±0.70	119.315	<0.001*
Range	20-300	5.6-8.1		

*: Highly significant

Table (2): Comparison between patients and control groups according to Total sperm number x (10⁶/ml).

T. Number x (10 ⁶ /ml)	Patients	Control	t-test	p-value
Mean±SD	12.80±4.12	24.30±6.56	88.061	<0.001*
Range	5-24	7-38		

*: Highly significant

Table (3): Comparison between patients and control groups according to Total sperm number x (10⁶/ml).

T. Number x (10 ⁶ /ml)	Patients	Control	t-test	p-value
<i>Mean±SD</i>	12.80±4.12	24.30±6.56	88.061	<0.001*
<i>Range</i>	5-24	7-38		

*: Highly significant

Table (4): Comparison between patients and control groups according to the percentage of Progressive Motility of sperms.

Prog. Motility (%)	Patients	Control	t-test	p-value
<i>Mean±SD</i>	16.38±9.87	72.63±8.40	753.722	<0.001*
<i>Range</i>	0-40	35-80		

*: Highly significant.

Table (5): Comparison between patients and control groups according to the percentage of Sperm DNA Fragmentation.

Sp. DNA. Frag (%)	Patients	Control	t-test	p-value
<i>Mean±SD</i>	34.88±14.65	15.25±5.88	61.801	<0.001*
<i>Range</i>	15-90	5-25		

*: Highly significant.

Table (6): Comparison between patients and control groups according to leukocytes count x (10⁶/ml).

Leukocytes x10 ⁶ /ml	Patients	Control	t-test	p-value
<i>Mean±SD</i>	1.59±1.36	0.45±0.21	27.261	<0.001*
<i>Range</i>	0.1-4.2	0.1-0.9		

*: Highly significant.

Table (7): Comparison between patients and control groups according to varicocele grade.

Varicocele / Grade	Patients		Control		Chi-square test	
	No.	%	No.	%	x2	p-value
0	28	0.70	36	0.90	9.200	0.027*
I	2	0.05	3	0.08		
II	3	0.08	1	0.03		
III	7	0.18	0	0.00		
Total	40	1.00	40	1.00		

*: significant.

Table (8): Relation between antisperm antibodies with TNF (pg/ml) in patients group.

TNF pg/ml	Anti. Sp. Ab		t-test	p-value
	Positive	Negative		
<i>Mean±SD</i>	113.0±62.7	140.5±76.3	1.055	0.311
<i>Range</i>	50-A220	20-A300		

Table (9): Correlation between TNF and other parameters, using Pearson Correlation coefficient in the patients group.

	TNF pg/ml	
	r	p-value
T. Number x (10 ⁶ /ml)	-0.703	<0.001
Prog. Motility (%)	-0.782	<0.001
Sp. DNA. Frag (%)	0.678	<0.001
Leukocytes x10 ⁶ /ml	0.839	<0.001
Varicocele Grade	-0.690	<0.001

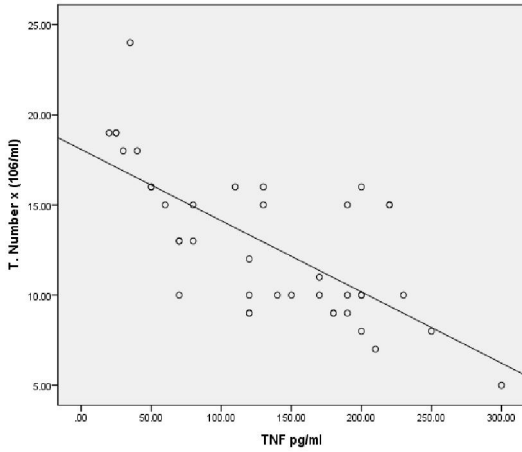


Fig. (1): Scatter plot between TNF level and T-Number.

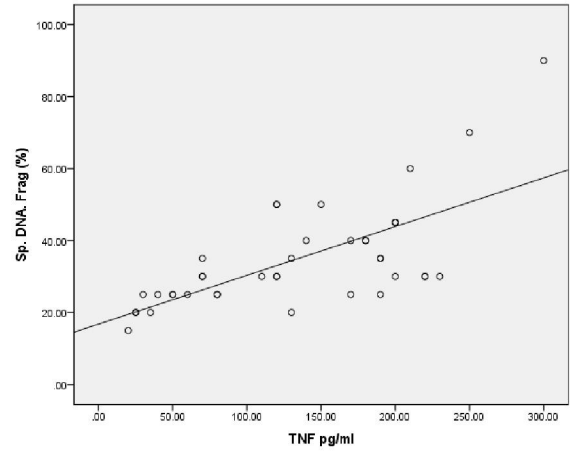


Fig. (3): Scatter plot between TNF level and Sp. DNA Frag. (%)

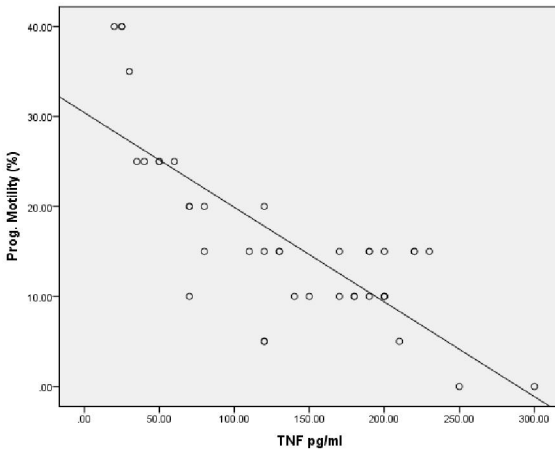


Fig. (2): Scatter plot between TNF level and Progressive Motility (%)

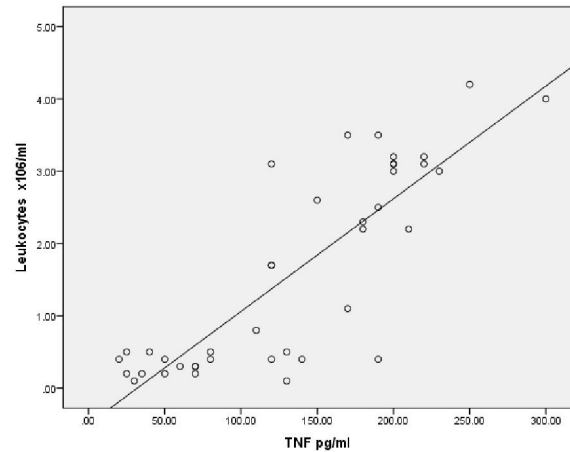


Fig. (4): Scatter plot between TNF level and leukocytes count.

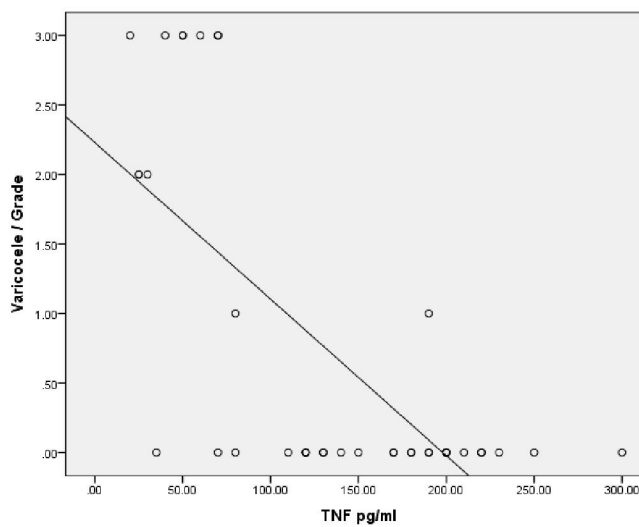


Fig. (5): Scatter plot between TNF level and varicocele grade

4. Discussion:-

Genital inflammation with or without infection, varicocele and anti-sperm antibodies etc., have been shown to have a common role in secondary infertility. Recently TNF had been revealed as a cytokine to have direct relation to male fertility. **(11).**

The role pathological TNF levels in relation to sperm parameter including DNA fragmentation is the value of our study.

From our study TNF was significantly increased in infertile groups compared to control group followed by a significant increase in sperm DNA fragmentation. This results is more or less coincide with the results of **(12)** that they show sperm nuclear DNA fragmentation is highly correlated with seminal TNF- α levels.

A significant decrease in sperm motility and count has been observed with supra-physiological TNF- α levels. These results were correlated with **(13).**

From our results TNF was significantly increased in inflammatory group compared to control group also TNF was significant increased in seminal leukocytes. These results were coincide with **(14).**

The most abundant population of white blood cells in seminal plasma are polymorphnuclear leukocytes. Studies havehypothesised that PMNs are able to release elastase PMN elastase), cathepsin G and collagenase. Elastase is secreted by azurophilic granules of neutrophil leukocytes during phagocytosis, degranulation or cell death and it may also lead to proteolytic damage of spermatozoa. PMN elastase is considered to be a first line sensitive and specific marker of silent male genital tract inflammation. In particular, due to the relatively rapid reaction of extracellularly liberated elastase with its major inhibitor, the α 1-proteinase inhibitor (α 1-PI), the enzyme can normally be detected in body fluid only in an inactive, complexed form, studies haveshown that the prevalence of high seminal elastase/diagnosis and prognosis of silent genital tract inflammation. The secretion of cytokines is part of the network of chemical reactions responsible for the inflammatory process, including TNF- α .**(15)**

Pro-inflammatory cytokines was revealed to be a natural component of seminal plasma, together with a broad array of immunologic factors, and they are involved in the physiological function of the male gonad, also acting in the fertilization process.**(16)**

Various cells in the male genitourinary tract release Cytokines and their production increases in response to foreign antigens, pathogens and chronic inflammations.**(17)**

Many authors have observed correlations between pro- inflammatory cytokine concentrations and the number of leukocytes, and the presence of leukocytes is inseparably associated with ROS production. For instance, the addition of interleukin

(IL-1 α), IL-1 β , TNF- α causes an increase in ROS production by human sperm concomitant with an increase in sperm membrane lipid peroxidation (LPO). The LPO of sperm plasma membrane can occur with TNF- α , either alone or in the presence of leukocytes**(18).**

Abdulridhaand Abid (19) observed an increased level of TNF- α in seminal plasma from individuals with genitourinary infections, suggesting that an elevated expression of TNF- α may be used as a diagnostic marker in male genital tract infections. Increased concentrations of TNF- α , have been reported to negatively influence sperm parameters.

Increase DNA fragmentation and decreased count/ motility with increased TNF- α was observed in our study, also our study showed a significantly increased TNF- α levels with leukocytospermia/pyospermia along with disturbed seminal parameters.

Dueto the vital role of apoptosis during spermatogenesis, in specific, at the stage of the development of stem spermatogonia into mature spermatozoa, much attention has been focused on the possible role of apoptosis regulators in pathologies associated with quantitative and qualitative deficits of spermatozoa**(19).**

TNF- α plays a key role in regulating apoptosis through binding with the type 1 TNF- α receptor (TNFR1). The binding of TNF- α to this receptor leads to the trimerization and recruitment of adaptor proteins, TRADD or FADD, through homophilic interactions between the conserved death domains (DD). FADD further recruits caspase-8 and causes its activation. Activated Caspase-8 can cleave Bid, a BH3-domain and Bcl-2 family protein, to form an active fragment, tBid. tBid interacts with Bax-Bak and activates the mitochondria permeability transition for the release of the cytochrome c. Following cytochrome c release, a high molecular-weight complex consisting of apoptotic protease activating factor-1 (APAF-1), cytochrome c, and caspase-9 is formed, which, in turn, activates a major execution caspase, caspase-3, followed by cell death**(20).**

The proapoptotic effect of TNF- α can also be mediated by ROS production. ROS can be generated in the endoplasmic reticulum and nuclear and plasma membranes. But mitochondria are the predominant source of ROS. ROS may promote cytochrome c release by inducing mitochondrial cristae reorganization and lipid peroxidation. This is very important because it is shown that elevated ROS production by spermatozoa in the male reproductive tract and ejaculate is associated, respectively, with oligozoospermia and the presence of chromatin degradation in mature spermatozoa. In addition, ROS cause peroxidative damage to the sperm plasma membrane. DNA

fragmentation in ejaculated spermatozoa has been correlated with oxidative stress (endogenous generation or exogenous stimulus) and with impaired sperm functional competence, including poor fertilization rates in IVF. ROS are also known to attack DNA, inducing strand breaks and other oxidative-based damage in human spermatozoa. (21)

This indicates that by binding to TNFR1, TNF- α activates several transduction pathways leading to regulation of the testicular expression of several genes involved in spermatogenesis. Said and colleagues (22) showed that TNF- α impairs functional and genomic integrity of spermatozoa in a manner that is reversible by the anti-TNF- α monoclonal antibody infliximab. (22)

Fertility is greatly affected by apoptosis-mediated sperm cell death. High apoptotic sperm rate may cause reduced sperm count or oligozoospermia. It has been revealed that VC, which is characterized by abnormally dilated veins in the pampiniform plexus causes haematologic testicular disorders as tissue hypoxia and the production of metabolic toxins such as free radicals, thereby increasing the presence of many apoptosis-inducing factors. (21)

Our research shows that in patients with VC, the apoptotic sperm rates and sperm densities have significant negative correlations with VC grades while seminal TNF- α more or less close to control group. It may be that an increased apoptotic sperm rate is an important cause of oligozoospermia.

Increased ROS and NO levels affect spermatozoon function in humans, which is mediated by the immune-modulators tumour necrosis factor- α (TNF- α) and interleukin 6 (IL-6). Studies by Ozdamarand colleagues (23) showed that high ROS levels in seminal plasma can impair the sperm cell membrane and cause damage to DNA, thereby triggering the start of apoptosis programmed cell death and increasing sperm apoptosis rates. Ozdamarand colleagues (23) found that VC can generate high levels ROS. They also showed that ROS levels in seminal plasma were significantly higher in patients with VC compared with the normal value range.

Wang and colleagues (24), found that the seminal plasma TNF- α levels in patients with VC were not higher than those in the control group, and that seminal TNF- α and leptin levels are unrelated in these patients. Therefore, he hypothesised that leptin promotes sperm apoptosis in VC patients via an unknown pathway that is not immune-mediated. Although this result different from our results could be due to the author Wang and colleagues (24) recruited patients with low grades varicocele.

In contrast to VC, the inflammatory and immune responses in leukocytospermia patients are quite

pronounced, suggesting that TNF- α is an active and important cytokine involved in this process.

From our research we can hypothesise that hypoxia is the etiological cause of varicocele and this sperm apoptosis. Also this apoptosis was correlated with the severity of varicocele ROS production secondary to hypoxia. This hypothesis was supported by the work of Wang and colleagues (24). He clarified the pathological association with male subfertility and an increase in intratesticular and seminal ROS levels in varicocele, and it has been associated with abnormalities in semen analyses. (24)

An increase in anti-sperm antibodies positively correlated with IL-6 and IL-8. The concentration of peroxidase-positive cells significantly correlated with an increase in IL-6, IL-8, TNF- α , and dead sperm. In addition, leukocytospermia was associated with a decrease in the sperm count, sperm progressive motility, and sperm normal morphology. (25) **تعداد الصياغة**

There is an indirect association between the antioxidant activity TNF- α and anti-sperm antibodies. We believe that there are many factors affecting sperm function. In seminal infection, the sperm dysfunction is most likely mediated through reactive oxygen species (ROS) generated by polymorphonuclear cells that affect the sperm cells by enhancing phagocytosis and the ROS produced by spermatozoa. (26)

Supra-physiologically high levels of TNF- α , IL-6, and IL-8, and anti-sperm antibodies were associated with declining seminal volume. Injury to tissues caused by infection/inflammation may damage the functioning of the accessory glands. Impaired secretions of the seminal vesicles results in a decreased volume of ejaculated and decrease of fructose concentration in seminal plasma. (16)

The presence of leukocytes in the semen highly correlated with a decrease in the quality of the sperm parameters such as sperm number, sperm motility and normal morphology, and increase of dead sperm. On the other hand, the leukocytes were associated with increase in pro-inflammatory molecules (IL-6, IL-8 and TNF- α). It has been evaluated that leukocytes can exert their negative effects on sperm by enhancing reactive oxygen species production. Moreover, the presence of pro-inflammatory cytokines has been revealed to be associated with an increase in peroxidation of the sperm membrane. (25)

Anti sperm antibody

In conclusion TNF had positive correlation with infertility, both sperm number and progressive motility and sperm DNA fragmentation had a direct correlation with TNF levels

Recommendation

Anti-TNF therapy is a new line of treatment

recommended to enhance fertility with patients suffering defective seminal parameters. Sperm Nuclear DNA fragmentation should be carefully considered with seminal analysis.

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