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Effect of Leukocyte Counts in Seminal Plasma on DNA Fragmentation of Spermatozoa

DALIA M. MOSTAFA, Ph.D.[4]; MANAL EL-HAMSHARY, Ph.D.[2]; AHMED M. BAHAA ELDIN, M.D.[3] and OMAIMA KHAMISS, Ph.D.[1]

Cell Culture and Molecular Virology, Genetic Engineering & Biotechnology Research Institute, University of Sadat City[1]
Molecular Diagnostic Department, Genetic Engineering & Biotechnology Research Institute, University of Sadat City[2]
Obstetrics & Gynecology Department, Faculty of Medicine, Ain Shams University[3] and Obstetrics & Gynecology Department, Faculty of Medicine, Ain Shams University and M.Sc. Candidate in Reproduction Technologies Genetic Engineering and Biotechnology Research Institute, University of Sadat City[4]

Abstract

Background: Infections of the male genitourinary tract account for up to 15% of cases of male infertility. Leukocytospermia is the presence of leukocytes in the semen, and the presence of >1 million leukocytes per mL of semen is considered as pathological.

Aim of Study: To investigate the effect of leukocyte count on DNA fragmentation percent.

Patients and Methods: The study included one hundred male participants from Zagazig City, Sharkiya, Governorate, Egypt in El-Banoon fertility Center from February 2019 to February 2023. Patients were divided into two groups: Group I control group consisting of 50 healthy males with normal semen analysis and Group II consisting of 50 males had leukocytes in the semen >1 million per mL.

Results: There was no significant difference between the 2 groups as regard age and BMI. Percentages of viscous samples and semen liquefactiontime had significantly higher and Percentages of progressive and total sperm motility had significantly lower in study group as compared to control group. leukocyte concentration and DNA Fragmentation were significantly higher in study group than control group while Viability was significantly lower in study group in comparison with control group. The study group had significantly lower sperm dynamic motility parameters including VCL, VSL, VAP, MAD, ALH and STR as compared to the control group. BCF and WOB were significantly higher with study group indicating a negative effect on linearity.

Conclusion: The present study suggested that bacterial infection significantly correlated with leukocytospermia, decreasing sperm motility, morphology, and DNA integrity.

Key Words: *Leukocytes count – Seminal plasma – DNA fragmentation of spermatozoa.*

Correspondence to: Dr. Dalia M. Mostafa, Obstetrics & Gynecology Department, Faculty of Medicine, Ain Shams University and M.Sc. Candidate in Reproduction Technologies Genetic Engineering and Biotechnology Research Institute, University of Sadat City

Introduction

UP to 15% of male infertility cases are caused by infections of the genitourinary tract. The function of sperm cells and the entire spermatogenetic process may be compromised by acute and chronic infections and the inflammation that results in the male reproductive system, leading to changes in the quality and quantity of sperm **[1]**. Leukocytes in semen are known as leukocytospermia, and more than one million leukocytes per milliliter of semen are regarded as pathological. In average, 10%–20% of people have pathological leukocytospermia; infertile men are more likely to have this condition **[2]**. There is conflicting information about leukocytes' function in semen. It has been reported that leukocytes in semen are physiological, serving as a surveillance system and phagocytosing aberrant and dead sperm **[3]**. According to recent research, leukocytes have a significant role in the generation of reactive oxygen species (ROS) in semen. Through the stimulation of cytokines and ROS formation in the seminal plasma, leukocytospermia can affect sperm function. The elevated ROS level is linked to lipid peroxidation in the sperm plasma membrane and can cause sperm DNA fragmentation **[4]**. Furthermore, an excess of reactive oxygen species (ROS) and the free radicals that follow in the seminal plasma damage proteins, RNA, or DNA, impede mitochondrial action, or peroxide the sperm's lipid component **[5]**.

According to a number of studies, leukocytospermia may play a major role in male infertility by impairing sperm motility and forward development or by increasing sperm DNA fragmentation, which may impact sperm fertilization capacity and potentially embryo quality **[6]**. Numerous investigations into the relationship between reproductive outcomes and DNA integrity have discovered that elevated levels of sDF lower the likelihood of becoming pregnant naturally, following intrauterine insemination (IUI), in vitro fertilization (IVF), and intracytoplasmic sperm injection (ICSI). Furthermore, sperm DNA damage is linked to a higher rate of miscarriages in IVF/ICSI cycles and recurrent miscarriages in aided and natural reproduction **[7]**. Therefore, sperm DNA damage detection can supplement the data from semen analysis **[8]**, so the current study aimed to investigate the prevalence rate of bacterial infection in subfertile men and its effect on DNA fragmentation percent.

Patients and Methods

Semen samples were collected from 100 male partners of the subfertile couples (mean age: 33.6± 10.1) attending in fertility centre between Feb. 2019 and April 2023. They were divided into 2 groups: Group I: Control groupconsisting of 50 healthy males with normal semen analysis and Group II: Consisting of 50 males had leukocytes in the semen >1 million per mL. A subfertile couple was defined as a couple who failed to achieve a clinical pregnancy after one year of regular andunprotected sexual intercourse.

Inclusion criteria: All patients had no colds and no history of special medications in the past 3 months (such as taking anti-oxidants and drugs affecting the immune systems).

Exclusion criteria: Excluding patients with abnormal testicular, epididymis, and vas deferens and azoospermia cases were also excluded.

All the patients provided the written informed consent.

All patientswere subjected to the following: Clinical evaluation and Investigations (Semen collection and Routine semen analysis).

Methods:

Sperm chromatin structure assay: Measure of DNA fragmentation percent by SCD technique byhalo sperm fragmentation kit.

The SCD Technique: Which is patented by Halotech is the foundation of halo sperm. It involves a carefully regulated denaturation of DNA to make it easier to extract the proteins that are present in each spermatozoon later on. In this sense, healthy spermatozoa produce halos made of DNA loops at the sperm head, which are absent from sperm with damaged DNA.

Semen preparation: Ejaculates were subjected to a standard andrological study, which included a leukocyte count and semen culture, following the 30 minutes of semen liquefaction at 37°C. We conducted analysis based on the guidelines provided by the World Health Organization (WHO) for volume, pH, concentration, motility, and morphology.

Leukocytospermia determination: According to WHO criteria by using peroxidase stain to detect granulocytes. The working solution is prepared by combining 1ml of saturated NH4Cl solution, 1ml of 5% of Na2 EDTA solution, 9ml of Ortho toluidine solution and 1 drop of $_{H2O2}$. This solution is mixed before use and can be conserved for 24h after preparation. A leukocytospermic condition was classified as having more than 1×10^6 /ml of semen.

Procedure:

Reagent Composition and Preparation: An SCD test was performed using the Halo sperm kit (INDAS Laboratories, Madrid, Spain).

Steps: An aliquot of each sperm sample at room temperature (22°C) was diluted to 5 to 10 million/mL in phosphate-buffered saline (PBS). Gelled aliquots of low melting point agarose. in Eppendorf tubes, were provided in the kit to process each semen sample. The Eppendorf tube was first placed in a water bath at 90°C to 100°C for 5 minutes to fuse the agarose and then in a water bath at 37°C for 5 minutes. 25µl of the diluted semen sample was added to the Eppendorf tube and mixed with the fused agarose. 14 to 20µl of the semen–agarose mixture was pipetted onto an agarose precoated slide (provided in the kit) and covered with a coverslip. The slide was then placed on a cold plate in the refrigerator (4°C) for 5 minutes for the agarose to produce a microgel, which could trap the sperm cells on the slide. An acid solution was then prepared by mixing 80µl of HCl from an Eppendorf tube in the kit with 10mL of distilled water. The coverslip was gently removed, and the slide was immersed horizontally in the acid solution and incubated for 7 minutes. Afterward, the slides were again incubated in 10mL of the lysing solution for 25 minutes and then in a bath of distilled water. The slides were dehydrated in 70%, 90%, and 100% ethanol baths for 2 minutes each and air-dried.Finally, the slides were horizontally covered with a mixture of eosin solution and Azur B solution for 6 minutes each. As a result, a minimum of 100 spermatozoa per sample were scored under the ×20 and ×40 objectives of a bright-field microscope.

Determination of Sperm DNA Fragmentation Ratio by Halo sperm Technique:

The spermatozoa were scored on each slide. A minimum of 100 spermatozoa per sample were

scored under the ×20 and ×40 objectives of bright-field microscope, and the total percentage of spermatozoa with DNA fragmentation including sperm cells with very small-sized halo, sperm cells without halo, and degraded sperm cells was calculated. Fig. (1) shows the five patterns of DNA Fragmentation.

Statistical analysis: Data was collected, coded, tabulated and introduced to the statistical package for social science (SPSS v 20.0).

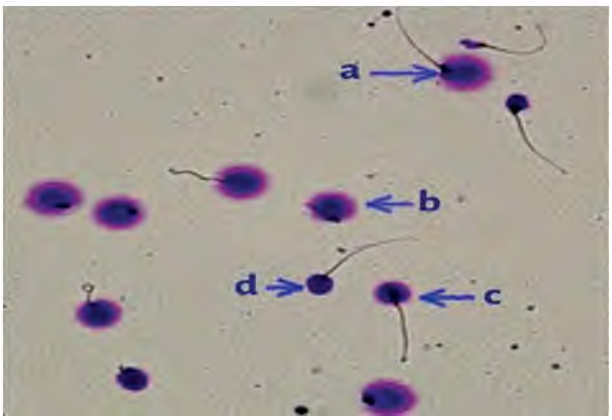

Fig. (1): Show Sperm DNA Fragmentation pattern. a- Big halo. b- Medium size halo, c- Small halo. d- Without halo.

Results

The median age of study group was 35 years while the median age of control group was 31 years, on the other hand the mean BMI for control group was 29, the mean BMI for study group was 28 there was no significant difference between the 2 groups as regard age and BMI (Table 1).

Percentages of viscous samples and semen liquefactive time had significantly higher in study group and progressive and total sperm motility Semen leukocytic count (mil/ml) Range had significantly lower in study group as compared to control group, while Semenvolume (ml) Range, Sperm count (mil/ejaculate) *Range, Normal sperm morphology% Range had no significantly difference (Table 2).

The study group had significantly lower sperm dynamic motility parameters including VCL, VSL, VAP, MAD, ALH and STR as compared to the control group. BCF and WOB were significantly higher with study group indicating a negative effect on linearity (Table 3).

Leukocyte concentration and DNA Fragmentation were significantly higher in study group than control group while Viability was significantly lower in study group in comparison with control group (Table 4).

Positive bacterial infection was present in 28 cases of Leukocytospermia and in 27 cases of fragmentation negative bacterial infection was present in 22 cases of Leukocytospermia and in 23 cases of fragmentation. This table revealed a significant positive Correlation of seminal bacterial infection with Leukocyto-spermia and DNA fragmentation in study group (infected group) (Table 5).

Table (1): Demographic criteria of the study groups.

Demographic criteria	Study group (n=50)	Control group (n=50)	Test of sig.	*p*
Age (years):				
Mean ± SD	35.0±8	31.0±9	MW	0.7
Range	20-37	20-38	90.0	
BMI (Kg/m^2):				
Mean ± SD	29.0±2	28.0±2	MW	0.8
Range	24-32	19-35	93.5	

Table (2): Semen variable of the studied groups.

Semen variable	Study group (n=50)	Control group (n=50)	*p*
Semen volume (ml):			
Range	1.1–6.50	1.0–8.1	>0.05
Mean ± SD	2.62±1.41	2.55±1.66	
Liquefactive time (min):			
Range	21–61	20.1–46	<0.01
Mean ± SD	40.00±12.751	28.61±8.10	
Percentages of samples viscosity (%)	61	28	<0.05
*Sperm count (mil/ejaculate) *:*			
Range	5.2–259	3.1–238	>0.05
Median	66	61	
Mean ± SD	81.91±87.261	72.63±85.1	
Normal sperm morphology %:			
Range	3–36	0–29	>0.05
Mean ± SD	13.44±9.19	15.00±6.99	
Progressive sperm motility %:			
Range	12–37	20–50	<0.001
Mean ± SD	22.04±8.67	39.08±11.41	
Total sperm motility %:			
Range	13–48	24–61	<0.001
Mean ± SD	26.80±11.03	45.44±13.16	
Semen leukocytic count (mil/ml):			
Range	2–9	0.1–0.7	<0.001
Mean ± SD	4.52±2.49	0.33±0.17	

Table (3): Comparison of dynamic sperm motility parameters between studied groups.

Semen variable	Study group (n=50)	Control group (n=50)	*p*
Curvilinear velocity (VCL) (µm/sec):			
Range	22–45	31–65	<0.001
Mean ŧ SD	36.09 ŧ6.01	55.95ŧ9.02	
Straight line velocity (VSL) (µm/sec):			
Range	8–31	26–44	<0.001
Mean ŧ SD	24.13ŧ5.29	38.09±5.75	
Average path velocity (VAP) (µm/sec):			
Range	11–35	28–47	<0.001
Mean ŧ SD	27.29±5.18	40.89±5.46	
Mean angular displacement degree (MAD):			
Range	35–67	32–64	<0.05
Mean ŧ SD	46.33±8.48	53.01ŧ10.91	
Lateral head displacement amplitude (ALH) (µm):			
Range	0–5.0	1–5.2	<0.05
Mean ŧ SD	2.61ŧ1.42	3.11±0.891	
Beat cross frequency (BCF) (Hz):			
Range	4.01–5.5	3.1–5.2	<0.01
Mean ŧ SD	4.45ŧ0.52	4.01ŧ0.51	
Linearity (VSL/VCL):			
Range	38.1–76.01	59.01–85.02	>0.05
Mean ŧ SD	66.56±8.45	69.68±6.97	
Oscillation (VAP/VCL):			
Range	52–84	63–89	<0.05
Mean ŧ SD	75.49 ŧ6.70	74.85±6.48	

Table (4): Semen Leukocytospermia, DNA Fragmentation and Viability of the studied groups. According to DNA Fragmentation levels.

Semen variable	Study group (n=50)	Control group (n=50)	*p*
Leukocytospermia (10^6/mL)	5.44 ±5.48	1.84 ± 1.94	>0.05
DNA Fragmentation (%)	42.24ŧ19.34	14.34 ŧ10.94	<0.01
Viability (%)	55.94ŧ13.54	85.04 ± 13.14	<0.05

- 15% or less fragmentation – excellent sperm DNA integrity.
- 15-to 25% DFI – good to fair.
- 25 – 50% DFI – fair to poor.
- 50% or greater DFI – extremely poor sperm DNA integrity.

Table (5): Correlation of seminal bacterial infection with Leukocytospermia and DNA fragmentation in study group (infected group).

Bacterial infection	Study group (N=50)		OR (95%CI)	*r*	*p*-value
	Leukocyto-spermia	DNA fragmentation			
Positive	5.44±5.48	1.84±1.94	3.98 (2.06–7.67)	+0.4	<0.001
Negative	42.24ŧ19.34	14.34ŧ10.94	42.24ŧ19.34	+0.4	
Total	55.94ŧ13.54	85.04±13.14			

Discussion

Up to 15% of male infertility cases are caused by infections of the genitourinary tract. The function of sperm cells and the entire spermatogenetic process may be compromised by acute and chronic infections and the inflammation that results in the male reproductive system, leading to changes in the quality and quantity of sperm **[1].**

According to recent research, the mere existence of bacteria in semen samples can lower the quality of the sperm. The bacteria that cause semen contaminations typically come from the patient's urinary system or can be passed from partner to partner during sexual activity **[3]**. Escherichia coli is the most often isolated microbe in male patients with genital tract infections or semen contamination. As shown at the ultrastructural level, this species' detrimental effects on motility and compromised acrosomal activity contribute to the poor quality of sperm **[9].** There is ongoing controversy regarding the connection between male infertility and bacterial infections and semen pollution. The fact that germs are just as common in infertile men's semen samples as they are in fertile men's does nothing but exacerbate the situation. It's still unknown what bacteria in semen mean clinically **[10].**

The results of our study were:

As regard Semenparametersof the studied groups: Percentages of viscous samples and semen liquefactive time had significantly higher in study group and progressive and total sperm motility percentages had significantly lower in study group as compared to control group.

According to certain research, bacterial infections cause the male urogenital tract to deteriorate **[10-13].** Nevertheless, additional research revealed that bacterial infection was unable to alter the characteristics of sperm., Hou et al., 2013 demonstrated that infection did not significantly alter the semen characteristics of fertile and infertile men in a very limited sample size **[13].** Patients with urogenital tract infections or semen infections have been shown to exhibit anomalies in sperm morphology. It has been demonstrated that sperm nuclear abnormalities brought on by an infected or inflamed urogenital tract are typically linked to poor sperm morphology. Research findings indicated that leukocytospermia and bacterial infection were responsible for the changed sperm morphology in semen contamination samples **[14]**. In the current study, all infected samples showed worse sperm morphology than non-infected samples. These results are in line with studies by Mehta et al., 2002 and Villegas et al., 2005 that found that sperm morphology is affected by infected semen **[15,16]**. Regarding Semen Leukocytospermia, DNA Fragmentation, and Viability of the Studied Groups, the current study found

that the Study Group had significantly higher Leukocyte Concentration and DNA Fragmentation than the Control Group, but the Study Group's Viability was significantly lower than that of the Control Group. An inflammatory disorder called leukocytospermia may be caused by an infection or inflammation in the semen **[17]**. Leukocytospermia is linked to numerous causes; nevertheless, we discovered a correlation between leukocytospermia and bacterial infection. This is because possible genital tract infections caused an inflammatory response that resulted in a rise in leukocytes in the seminal fluid **[18]**. Numerous investigations demonstrated that bacterial contamination could directly and significantly lower sperm quality in the semen analysis, regardless of leukocytospermia. For example, sperm agglutination may result from interactions between glycoproteins and receptors on the sperm surface and bacterial flagella, and some adhesion events may suggest a decrease in sperm motility **[12]**. Additionally, by lowering membrane potential and mitochondrial ATPase activity, soluble spermatotoxic substances like sperm immobilization factor affect sperm viability and motility **[6]**. Our results demonstrated that sperm viability was much lower in infected semen compared to non-infected, which is consistent with the earlier findings. Regarding this, Varela et al., 2018 shown that a load of microbial contamination in semen samples dramatically reduced the number of viable sperm **[19]**. Regarding the current study's correlation between DNA fragementation and seminal bacterial infection with Leukocytospermia, our analysis showed a statistically significant positive correlation between the two variables in the study group. Previous research found that sperm DNA fragmentation is caused by leukocytospermia as well as excessive reactive oxygen species (ROS) formation that occurs after bacteriospermia **[20]**. In fact, patients with infections of the urogenital tract may have elevated seminal ROS levels during bacterial infections **[13]**. Bacteria may have an impact on semen parameters because of their capacity to generate certain inflammatory mediators, recruit leukocytes, and ultimately elevate ROS. Sperm DNA fragmentation deteriorates when leukocytospermia and elevated ROS levels are present **[14]**. According to the current research, patients with the infection had considerably higher levels of sperm DNA fragmentation than those without it. Furthermore, we discovered a statistically significant association between sperm DNA fragmentation and leukocytospermia. According to Zeyad et al., 2018 sperm DNA condensation is negatively impacted by bacterial infection **[6]**. Additionally, Domes et al., 2012 found a strong association between leukocytospermia and DNA fragmentations, which is in line with the current findings. All things considered, it appears that bacterial infection can impact DNA integrity in patients with seminal infection by inducing inflammatory mediators **[21]**.

Conclusion:

Male infertility can result from a variety of unique circumstances. According to this study, leukocytospermia in infertile men may be a predictor of seminal bacterial contamination. Our findings showed that semen parameters are negatively impacted by bacterial infection. Therefore, sperm DNA integrity may be impacted by leukocytospermia and bacteriospermia, which may also impair embryonic development and pregnancy outcomes. Treatment for urogenital tract infections appears to be beneficial in increasing the likelihood of conception in infertile males. Additional research on this subject is necessary.

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تأثير عدد كريات الدم البيضاء فى البلازما المنوية على تكسير الحمض النووى للحيوانات المنوية

تمثل التهابات الجهاز البولى التناسلى عند الذكور ما يصل إلى ١٥٪ من حالات العقم عند الذكور. زيادة عدد الكريات البيض فى الدم هو وجود كريات الدم البيضاء فى السائل المنوى، ويعتبر وجود أكثر من مليون خلية دم بيضاء لكل مليلتر من السائل المنوى من الحالات المرضيَّة.

الهدف من هذه الدراسة تأثير عدد الكريات البيض فى السائل المنوى على نسبة تكسير وتدمير الحمض النووى.

شملت الدراسة مائة مشارك من الذكور من مدينة الزقازيق، محافظة الشرقية، مصرفى مركز البنون للخصوبة وأطفال الأنابيب في الفترة من فبراير ٢٠١٩ إلى فبراير ٢٠٢٣. تم تقسيم المرضى إلى مجموعتين: المجموعة الأولى، مجموعة مراقبة تتكون من ٥٠ ذكرًا سليمًا مع تحليل طبيعى للسائل المنوى والمجموعة الثانية تتكون من ٥٠ ذكرًا من الحالات التى يتوفر فيها وجود كريات الدم البيضاء فى السائل المنوى اكثر من مليون لكل مليلتر. اثبتت النتائج انه لم يكن هناك فرق كبير بين المجموعتين فيما يتعلق بالعمر ومؤشر كتلة الجسم، بينما كانت النسب المئوية للعينات اللزجة (درجة لزوجة السائل المنوى) ووقت تسييل السائل المنوى أعلى بكثير وكانت النسب المئوية لحركة الحيوانات المنوية التقدمية (Motility) والإجمالية أقل بكثير فى مجموعة الدراسة مقارنة بالمجموعة الضابطة (التى يمثلها العينات المقارنة او السليمة) . كما لوحظ انه كان تركيز كريات الدم البيضاء وتفتيت الحمض النووى أعلى بكثير فى مجموعة الدراسة مقارنة بالمجموعة الضابطة بينما كانت درجة حيوية العينات (Viability) أقل بشكل ملحوظ فى مجموعة الدراسة مقارنة بالمجموعة الضابطة. بالإضافة الي انه كان لدى مجموعة الدراسة معلمات حركة ديناميكية للحيوانات المنوية أقل بشكل ملحوظ بما فى ذلك VCL وVSL وVAP وMAD وALH وSTR مقارنة بالمجموعة الضابطة، و كان BCF وWOB أعلى بشكل ملحوظ حيث أشارت مجموعة الدراسة إلى وجود تأثير سلبى على الخطية.

الاستنتاج: أشارت الدراسة الحالية إلى أن العدوى البكتيرية ترتبط بشكل كبير مع زيادة عدد الكريات البيضاء فى الدم، وانخفاض حركة الحيوانات المنوية، وشكلها، وسلامة الحمض النووى.