

Impact of leukocytospermia on sperm dynamic motility parameters, DNA and chromosomal integrity

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Introduction To characterize sperm dynamic motility patterns and chromatin integrity in infertile men with leukocytospermia.

Material and methods Fifty patients with primary infertility and oligoasthenoteratozoospermia included in this prospective, controlled, blind study. All patients underwent clinical evaluation, semen peroxidase stain, computer aided semen analysis (CASA), sperm DNA integrity evaluation with acridine orange test (AOT) and fluorescence in situ hybridization (FISH) analysis of 18, X and Y chromosomes. Pregnancy outcomes were documented following antibiotic treatment of patients with leukocytospermia.

Results Infertile men with leukocytospermia had significantly lower progressive and total sperm motility percentages compared to the control group. Sperm dynamic motility parameters by CASA including curvilinear, straight line and average pathway velocities, straightness and amplitude of lateral head displacement were significantly lower in leukocytospermia. Sperm DNA fragmentation index was significantly higher in leukocytospermia. Percentages of sperm with disomy XY and 18 were significantly higher. These changes in sperm motility parameters and DNA integrity correlated with the number of peroxidase positive leukocytes. Follow-up of 23 of the 25 patients with leukocytospermia after antibiotic treatment revealed significantly higher pregnancy rates in cured patients than in those with persistent leukocytospermia.

Conclusions Leukocytospermia has a significant impact on sperm dynamic motility patterns, DNA and chromosomal integrity in infertile men which can adversely affect the likelihood of a successful pregnancy.

Key Words: leukocytospermia ↔ computer aided semen analysis ↔ sperm DNA ↔ FISH

INTRODUCTION

Leukocytospermia is defined by the World Health Organization (WHO) as $\geq 1 \times 10^6$ WBC/ml of semen [1]. An elevated leukocyte count is found in the seminal fluid of up to 30% of infertile men, even in the absence of inflammatory symptoms or a seminal bacterial infection [2]. Evidence from several studies indicates that leukocytospermia could significantly contribute to male infertility by decreasing sperm motility, increasing sperm DNA damage and affecting the fertilizing ability. The great majority of these studies

used manual semen analysis [3, 4, 5]. Fewer studies applied computer aided semen analysis (CASA) [6]. CASA technology has been applied widely in clinical andrology laboratories [7]. CASA helps provide precise and meaningful information about sperm motility fractions and dynamics, and carries out statistical analysis of the retrieved data [8].

Sperm DNA integrity is an objective parameter of sperm quality that correlates with both natural conception probability and the outcome of assisted reproductive procedures [9]. Despite some debate regarding its accuracy, acridine orange test (AOT)

is a simple and rapid test that is still applied in the evaluation of sperm DNA integrity of infertile men with different etiologies. Its results are comparable to the other more advanced tests such as TUNEL and flowcytometry [10, 11].

Sperm from infertile men may be genetically defective at the chromosomal level, containing numerical and/or structural chromosomal aberrations [12]. Fluorescence in situ hybridization (FISH) is the most commonly used tool to determine the proportion of aneuploidy present in sex chromosomes and autosomes of sperm from infertile men [13].

The current study aims to characterize sperm dynamic motility patterns and chromatin integrity in infertile men with leukocytospermia.

MATERIAL AND METHODS

Twenty-five infertile men with oligoasthenoteratozoospermia and leukocytospermia were consecutively recruited from the Andrology clinic, Department of Dermatology and Andrology, Assiut University Hospital. Another 25 men with oligoasthenoteratozoospermia and no leukocytospermia were included as a control group. The study has been approved by the institutional review board and all patients signed an informed consent form. Exclusion criteria were history of smoking, occupational risk of exposure to sperm DNA toxins, genital disorders that impair reproductive capacity such as clinical varicocele, undescended testis and testicular atrophy, systemic diseases that may impair reproductive capacity such as hepatic, renal, endocrine and autoimmune diseases, azoospermia, administration of antioxidants, or presence of female partner factor infertility contributing to infertility. All patients underwent history taking, and thorough general medical and genital examination. Semen collection was carried out after an abstinence period of 2–7 days, with initial conventional analysis as per WHO 2010 guidelines [14]. Assessment of leukocytic count was carried out using peroxidase stain (LeucoScreen test, FertiPro, Belgium) [4]. Patients with leukocyte concentration in semen equal to or more than $10 \times 6/\text{ml}$ were placed into the leukocytospermia group. Other patients with leukocytic concentration in semen less than $10 \times 6/\text{ml}$ were placed in the non-leukocytospermia group [15].

Semen analysis

Following the assessment of semen content of leukocytes, further investigations were done in a blinded manner; thus, researchers were unaware which sample belonged to which study group. The evalu-

ation of sperm concentration and motility was carried out using Mira-9000 CASA Quick Use system (MiraLab, Geneva, Switzerland). Different dynamic sperm motion parameters were assessed including curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), LIN or linearity (VSL/VCL), STR or straightness (VSL/VAP), WOB or oscillation (VAP/VCL), ALH or lateral head displacement amplitude, BCF or beat cross frequency and MAD or mean angular displacement degree. Sperm morphology was evaluated using Diff-Quik staining (Polysciences Inc., Warrington, PA) and sperm deformity and teratozoospermia indices (SDI and TZI) were determined [1, 14].

Acridine orange test [16]

The AOT assay measures the ability of sperm nuclear DNA to denature in acid which forms a meta-chromatic shift of AO fluorescence from green (native DNA) to red (denatured DNA). The fluorochrome AO intercalates in the double-stranded DNA as a monomer, which binds to single-stranded DNA. The monomeric AO bound to native DNA fluoresce green, whereas the aggregated AO on denatured DNA fluoresces red. By using fluorescent microscopy, thick semen layers are fixed in fixative (methanol: acetic acid 3:1) for 2 hours. The slides are stained for 5 minutes and rinsed with water. The slides were washed with distilled water then covered with a glass cover and examined under a ZEISS mot plus (ZEISS, Germany) fluorescent microscope at the excitation wavelength of 450–490 nm. An average of 200 sperm cells was evaluated on each slide by the same examiner. Spermatozoa showing green fluorescence were interpreted as having a normal DNA content, whereas those displaying a spectrum of yellow-orange to red fluorescence were considered to have damaged DNA. The ratio between (yellow to red)/(green + yellow to red) fluorescence was considered as sperm DNA fragmentation index (DFI) percentages.

FISH analysis for sperm chromosomes 18, X and Y [17]

FAST FISH prenatal X, Y and 18 enumeration (Cytocell, Cambridge, UK) were carried out using a probe designed for the detection and quantification of these chromosomes in the sperm nuclei. FISH analysis involved hybridization of chromosome-specific DNA probes labeled with fluorochromes to complementary DNA sequences on target chromosomes, followed by detection of the bound probes under a fluorescence microscope. Spermatozoa were evaluated as normal when a single signal was detected for blue (chromosome 18) and either orange (Y chromo-

some) or green (X chromosome). The total number of signals for each chromosome were subsequently calculated and disomy, as well as nullisomy (loss) rates were calculated for X, Y and 18.

Statistical analysis

The data were analyzed and expressed as mean values \pm standard deviations using SPSS version 17 program (SPSS, Inc., IBM, Armonk, NY). Unpaired t-test was used in comparisons of numerical parametric data between different groups. The Mann-Whitney test was used in comparison of numerical non-parametric data between groups. Fisher's exact test was used to compare percentages. Spearman correlation test was applied to analyze correlations between different quantitative variables. Values were considered significant when P values were equal to or less than 0.05.

Pregnancy outcomes

Patients with leukocytospermia received proper antibiotic treatment and were contacted for a telephone interview in June 2017 to inquire whether or not their partners had become pregnant.

RESULTS

Baseline characteristics showed no significant statistical difference between men with and without leukocytospermia; mean age was 32.1 ± 6.4 years vs. 33.7 ± 7.7 years respectively, mean BMI was 22.0 ± 1.8 vs. 21.8 ± 2.1 kg/m², and mean duration of infertility was 4.2 ± 2.1 years vs. 5.6 ± 5.4 years respectively ($P > 0.05$).

Infertile men with leukocytospermia had significantly higher semen liquefactive time, and percentages of viscous samples. They also had significantly lower progressive and total sperm motility percentages as compared to the non-leukocytospermia group (Table 1).

The leukocytospermia group had significantly lower CASA sperm dynamic motility parameters including VCL, VSL, VAP, MAD, ALH and STR as compared to the non-leukocytospermia group. BCF and WOB were significantly higher with leukocytospermia indicating a negative effect on linearity (Table 2).

There were statistically significant increases in the percentages of the sperm DNA fragmentation index (DFI) by AOT, disomy XY, disomy 18, and the percentage of samples with disomy by FISH in the leukocytospermia group as compared to the non-leukocytospermia group (Table 3, Figure 1).

Significant negative correlations were found between the number of peroxidase positive leukocytes and

sperm motility parameters as evaluated by CASA, including progressive sperm motility ($r = -0.580$, $P < 0.001$), VCL ($r = -0.590$, $P < 0.01$), VSL ($r = -0.526$, $P < 0.01$), VAP ($r = -0.424$, $P < 0.05$). There was also significant positive correlation between semen leukocytic count and the percentage of sperm DFI ($r = 0.767$, $P < 0.001$).

The 25 patients with leukocytospermia received proper antibiotic treatment, 23 of them were successfully followed-up, and two patients were lost during follow up. Thirteen of the 23 patients were cured and 10 had persistent leukocytospermia. Five of the 13 cured patients' partners reported clinical pregnancy while 1 of the uncured 10 reported clinical pregnancy, which, unfortunately, ended in a spontaneous abortion.

DISCUSSION

Leukocytospermia is a common finding in the semen of infertile men [1]. Apart from being a sign of an accessory gland or genitourinary infection, it may also

Table 1. Comparison of physical and microscopic semen variables in leukocytospermia and non leukocytospermia group

Semen variable	Leukocytospermia group (n = 25)	Non leukocytospermia group, (n = 25)	P-value
Semen volume (ml)			
Range	1–6.5	1–8	
Mean \pm SD	2.61 \pm 1.40	2.56 \pm 1.67	>0.05
Liquefactive time (min)			
Range	20–60	20–45	
Mean \pm SD	39.00 \pm 12.75	28.60 \pm 8.10	<0.01
Percentages of viscous samples (%)	60 (15/25)	28 (7/25)	<0.05 χ^2
Sperm count (mil/ejaculate)*			
Range	5–258	3–239	
Median	64	61	
Mean \pm SD	81.90 \pm 87.26	72.63 \pm 85.00	>0.05
Normal sperm morphology%			
Range	3–36	0–29	
Mean \pm SD	13.44 \pm 9.19	15.00 \pm 6.99	>0.05
Progressive sperm motility %			
Range	12–37	20–50	
Mean \pm SD	22.04 \pm 8.67	39.08 \pm 11.41	<0.001
Total sperm motility %			
Range	13–48	24–61	
Mean \pm SD	26.80 \pm 11.03	45.44 \pm 13.16	<0.001
Semen leukocytic count (mil/ml)			
Range	1–8	0.1–0.8	
Mean \pm SD	3.52 \pm 2.49	0.34 \pm 0.18	<0.001

χ^2 Chi-square test

Table 2. Comparison of dynamic sperm motility parameters between both groups

Semen variable	Leukocytospermia group	Non leukocytospermia group	P-value
VCL ($\mu\text{m}/\text{sec}$)			
Range	21–44	32–64	
Mean \pm SD	36.08 \pm 6.00	55.96 \pm 9.01	<0.001
VSL ($\mu\text{m}/\text{sec}$)			
Range	8–30	25–45	
Mean \pm SD	24.12 \pm 5.28	38.08 \pm 5.74	<0.001
VAP ($\mu\text{m}/\text{sec}$)			
Range	10–34	27–46	
Mean \pm SD	27.28 \pm 5.17	40.88 \pm 5.45	<0.001
MAD			
Range	36–68	31–63	
Mean \pm SD	46.32 \pm 8.47	53.00 \pm 10.92	<0.05
ALH (μm)			
Range	0–5	1–5	
Mean \pm SD	2.60 \pm 1.41	3.10 \pm 0.89	<0.05
BCF (Hz)			
Range	4–5	3–5	
Mean \pm SD	4.44 \pm 0.51	4.00 \pm 0.50	<0.01
LIN			
Range	39–75	58–84	
Mean \pm SD	66.56 \pm 8.43	69.68 \pm 6.97	>0.05
WOB			
Range	51–83	62–88	
Mean \pm SD	75.48 \pm 6.72	74.84 \pm 6.47	<0.05
STR			
Range	62–90	83–96	
Mean \pm SD	85.52 \pm 6.04	91.56 \pm 4.06	<0.01

be associated with numerous other risk factors for male subfertility including smoking, obesity, autoimmune disorders, exposure to environmental pollution and defective spermatogenesis [18, 19].

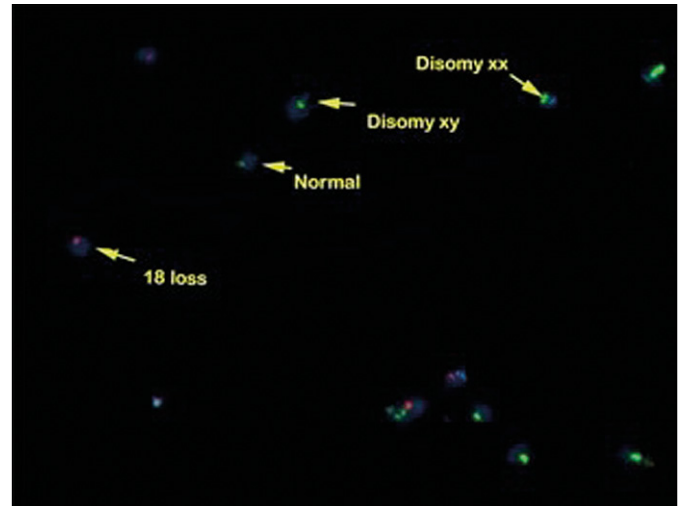
The changes in semen parameters in leukocytospermia may be a reflection of oxidative stress due to excessive ROS production by leukocytes, dysfunction of the male genital ducts and glands by an infectious process, or they are induced by the microorganisms causing an infection [20].

In our study, there was a statistically significant increase in semen hyperviscosity in the leukocytospermia group when compared with the non leukocytospermia group. This finding is in accordance with most previous studies reporting seminal hyperviscosity in leukocytospermia with different techniques [21, 22, 23]. Semen hyperviscosity is mostly attributed to male accessory gland infection that commonly associates leukocytospermia with inflammation, as well as dysfunction of the sex glands or even the immune system [21].

CASA provides an objective approach to routine manual semen analysis that can promote laboratory standardization and allows for more precise and de-

Table 3. Comparison of sperm DNA and chromosomal integrity between the two groups

Semen variable	Leukocytospermia group	Non leukocytospermia group	P-value
Sperm DFI (%)			
Range	25–70	20–35	
Mean \pm SD	43.60 \pm 13.43	30.20 \pm 5.10	<0.001
Disomy XY (%)			
Range	0–0.1	0–0.05	
Mean \pm SD	0.017 \pm 0.027	0.004 \pm 0.012	<0.05
Disomy XX (%)			
Range	0–0.1	30–70	
Mean \pm SD	0.009 \pm 0.025	50.1 \pm 9.68	>0.05
Disomy YY (%)			
Range	0–0.02	0–0.02	
Mean \pm SD	0.002 \pm 0.005	0.001 \pm 0.004	>0.05
Disomy 18 (%)			
Range	0–0.2	0–0.05	
Mean \pm SD	0.032 \pm 0.048	0.005 \pm 0.011	<0.05
Percentages of semen samples with disomy (%)	68% (17/25)	36% (9/25)	<0.05

**Figure 1.** Disomy XY, XX and 18 chromosome nullisomy by FISH in a case of leukocytospermia.

tailed analysis of sperm motion criteria [24]. CASA results in our study showed a significantly higher decrease in progressive sperm motility percentage, total sperm motility percentage and many CASA sperm dynamic velocities such as VCL, VSL, VAP, MAD, ALH and STR in the leukocytospermia group than in the non leukocytospermia group. Also, there were significant negative correlations between the number of leukocytes using the peroxidase stain and progressive sperm motility percentage, total sperm motility percentage, VCL, VSL and VAP. Meanwhile, BCF and WOB were increased in the leukocytospermia group. This increase in BCF (which refers to the

average rate at which the curvilinear path crosses the average path) and WOB (which refers to the oscillation of the actual path about the average path expressed as VAP/VCL) reflect the decrease in the sperm linearity. Since these dynamic motility patterns and velocities are important markers for sperm capacitation, the changes detected by CASA indicate that leukocytospermia may have a negative impact on important sperm functions. Our study is the first to characterize such changes in sperm dynamic motility patterns in leukocytospermia. Previous studies using CASA in leukocytospermia reported a significant negative correlation between the semen leukocyte count and the percentage of sperm motility but without a comment on other dynamic sperm motility parameters [25, 26]. On the other hand, another study found no significant difference in sperm motility between infertile patients with or without leukocytospermia [27]. Changes in sperm motility parameters detected by conventional semen analysis and CASA in leukocytospermia can be attributed to excessive ROS release, which causes peroxidation of lipids in sperm plasma membrane and dysfunction [20].

In our study, there was a statistically significant increase in the DFI in the leukocytospermia group as compared to the non-leukocytospermia group. Also there was a significant positive correlation between the number of pus cells by peroxidase stain and the sperm DNA fragmentation index by acridine orange test. Similarly, Saleh et al. [25], using the sperm chromatin structure assay (SCSA) to assess sperm DNA integrity reported a positive correlation between sperm DFI and leukocytospermia. A subsequent study reported a positive association between leukocytospermia and increased DNA fragmentation using the COMET technique [3]. More recently, Agarwal and associates measured sperm DNA damage using the TUNEL technique and found it to be significantly higher in the low leukocytospermia infertile group (0.1–10 x6/ml) as compared to the non leukocytospermia infertile group [27]. A later study also found a positive correlation between the number of seminal leukocytes and the number of spermatozoa with TUNEL-detected DNA fragmentation [26]. Leukocytospermia was hypothesized to play a major role in sperm DNA damage via oxidative stress and defective apoptosis [3]. On the other hand, an earlier study reported a very weak correlation between leukocytospermia and DNA fragmentation index measured with flow cytometry with acridine orange stain. The authors of this study mentioned that the

role of leukocytes as the main source of reactive oxygen species in the pathology of sperm DNA damage may be overestimated [28].

To the best of our knowledge, this is the first study that used the FISH technique to demonstrate sperm chromosomal aneuploidy in the semen of infertile patients with leukocytospermia. FISH analysis in our study showed significant increases in the percentages of sperm with disomy X, Y and 18 and percentage of semen samples with disomy in the leukocytospermia. A possible explanation for the positive correlation between leukocytospermia and both sperm DNA damage and chromosomal aneuploidy may be related to an association between leukocytospermia induced oxidative stress and defective spermiogenesis [25]. Defective spermiogenesis results in an increased production of sperm with defective nuclear and membrane remodeling. It has been postulated that men with reduced spermatogenesis may not produce enough spermatozoa to trigger Sertoli cells to produce Fas ligand that is thought to stimulate the apoptosis process and thus, these abnormal, damaged sperms escape apoptosis [29]. Our data suggest that at least some sperm aneuploidies are acquired and may result from inflammation and/or excessive ROS production. These results are important considering the serious genetic consequences of these genetic abnormalities on increased incidence of congenital anomalies in the offspring of affected males and necessitate certain intervention such as antioxidant supplementation, which has been reported to reduce the incidence of aneuploidy in men with severe oligoasthenoteratospermia [30].

CONCLUSIONS

Sperm from infertile men with leukocytospermia showed remarkable changes in its dynamic motility patterns, DNA and chromosomal integrity. Further studies on larger patient groups are required to validate our findings.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ETHICAL APPROVAL

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

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