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Chlamydia trachomatis Infection: its relation to semen parameters and sperm DNA integrity

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Abstract

The present study was conducted to determine the effect of *Chlamydia trachomatis* (CT) infection on semen quality and sperm DNA integrity. The study included 60 infertile male patients with CT infection and 25 age matched controls. Diagnosis of patients was based on detection of CT IgA by ELISA in seminal plasma and CT plasmid DNA in the semen sediment. All patients and controls were subjected to the following investigations: history taking, conventional semen analysis, detection of CT IgA, Plasmid DNA in semen samples, reactive oxygen species (ROS) and percentage of DNA fragmentation. There was significant increase in semen ROS levels and the percentage of sperm DNA fragmentation in the CT patient group when compared to the control group ($P<0.05$) and in those with leukocytospermia when compared to those without leukocytospermia ($P<0.05$). In the patient group with CT infection there was a positive correlation between the percentage of DNA fragmentation, ROS ($r = 0.82$ with $P<0.0001$) and pus cell count. ($r = 0.7$ with $P<0.0001$). In patients with leukocytospermia, there was a positive correlation between the percentage of DNA fragmentation, ROS ($r = 0.9$ with $P<0.0001$) and pus cell count ($r = 0.83$ with $P<0.0001$). In conclusion, sperm concentration, mobility, and viability, are significantly decreased in patients with CT compared to controls. ROS levels and the percentage of sperm DNA fragmentation significantly increased in CT patients especially patients with leukocytospermia.

Keywords: Chlamydia - Leukocytospermia, Semen parameters, and Sperm DNA integrity.

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Introduction

Chlamydia trachomatis (CT), an obligate intracellular bacterium, is requiring living cells for replication. It has a biphasic life cycle characterized by an elementary body (EB) and a reticular body that is able to replicate within eukaryotic cells. It is considered one of the most prevalent sexually transmitted diseases worldwide.[1] Men can harbor sub-clinical infections in the genital tract over extended periods of time. CT has been detected in semen from asymptomatic men.[2] In addition, chlamydia infection in men can cause urinary tract inflammation, sperm DNA damage, acute epididymitis, urethritis, prostatitis and orchitis.[3]

Several mechanisms in which *C. trachomatis* could affect semen quality including apoptosis of spermatozoa caused by chlamydial lipopolysaccharide and persistent infection resulting in scarring of the ejaculatory ducts. Damage in the epithelial cells involved in spermatogenesis can also impair sperm quality. CT's infection can persist for up to four years within couples and affect their fertility.[4] In men CT antigens are the best immunologic markers of chronic chlamydia prostatitis.[5]

The presence of leukocytes in semen samples is a marker of CT infection, and the World Health Organization (WHO) defines leukocytospermia as more than 1×10^6 WBC/mL.[6] Leukocytospermia has a negative impact on semen quality due to the production of reactive oxygen species (ROS). ROS produced by leukocytes increase apoptosis in mature human spermatozoa, more likely to evidence sperm with DNA fragmentation. The apoptotic mode of cell death includes, principally, plasma membrane externalization of phosphatidylserine (PS) and DNA fragmentation.[7] Accordingly, this study aimed to determine the effect of *C. trachomatis* infection on semen quality and sperm DNA integrity.

Materials and Methods

This case control study was performed on 60 infertile male patients with CT infection. Patients were selected out of 250 infertile patients from the Andrology Unit, Assiut University hospital. Twenty-five age matched infertile patient with negative chlamydia and no pyospermia served as a control group.

The protocol of the study was reviewed and approved by the Ethical Committee of the Faculty of Medicine, Assiut University (approval date: July 2021). An informed consent was obtained from all patients and controls. Diagnosis of patients was based on detection of CT IgA by ELISA in seminal plasma and CT plasmid DNA in the semen sediment. All patients and controls were subjected to the following investigations: history taking, conventional semen analysis, and detection of CT IgA. Plasmid DNA in semen samples, ROS and percentage of DNA fragmentation were performed for CT patients and the control group. Patients with varicocele, azoospermia, smokers, and those under drugs affecting spermatogenesis for at least the last three months prior to study were excluded from study.

Semen analysis

Semen samples were obtained by masturbation after an abstinence period of 3 to 5 days. The semen sample was analyzed macroscopically and microscopically for volume, viscosity, pH, sperm concentration, percentage of progressively motile sperm, sperms morphology, and number of round cells according to WHO (1999).[8] Liquefied semen specimens were centrifuged at 300 g for 7 min. Seminal plasma was removed and frozen at -20°C until used for detection of anti-CT IgA.

Leuco-Screen Test

Leuco-Screen test was performed on fresh semen sample using a commercially available Leuco-Screen kit (lot number FP09 I05 R01 B.13) supplied by FertiPro N.V, Belgium. The semi-quantitative histochemical commercial kit was used for the determination of peroxidase positive white blood cells (WBC) in human semen, according to the manufacturer's instructions. The principle of the kit is based on that granules in the polymorphonuclear WBC contain peroxidase. The peroxidase catalyzes hydrogen peroxide into water and free oxygen ions, which in turn, oxidize benzidine. Oxidized benzidine is brown in color and consequently, peroxidase-positive cells have a brown coloration. Reagent 1, supplied in the kit, contains a red contrast fluid which facilitates the differentiation between peroxidase positive round cells and peroxidase negative round cells.[9]

Measurement of semen ROS level

The ROS level was measured in fresh semen using a kit containing dihydro-1,4-phthalazinedione supplied by MP Biomedicals™ (Fishersci.fi., Finland). The principle is based on detecting the Chemiluminescence activity using the luminol solution. The semen pellet was washed twice with phosphate-buffered saline (PBS) pH 7.4 by centrifuge at 300 g for 5 min

and resuspended in PBS at a concentration of 20x10^6 sperm/ml then centrifuged and the supernatant was discarded. Ten ml luminol, used as a probe, was added to the tube containing the semen pellet. ROS levels were assessed by measuring chemiluminescence activity by Autolamat Luminometer (Berthold Technologies, Bad Wildbad, Germany) in the integrated mode for 15 min.

Detection of anti-CT IgA in seminal plasma

Detection of anti-CT IgA in patients' seminal plasma was performed on Alegeria, a fully automated Random-Access single-test ELISA analyzer, using a commercial kit supplied by ORGENTEC Diagnostika GmbH, Germany (lot number ORG 906A), based on an indirect enzyme-linked immunosorbent assay. Positive results were considered at > 25 U/ml.[10]

Detection of *C. trachomatis* plasmid DNA by the Polymerase Chain Reaction (PCR):

Sperms' DNA was extracted by using a commercially available kit (QIAamp DNA Mini Kit), supplied by Qiagen, Germany. Detection of *C. trachomatis* plasmid DNA was performed by PCR using primers sequence as follow: F (5`-TCC GGA GCG AGT TGA GAA GA-3`) and R (5`AAT CAA TGC CCG GGA TTG GT-3`). For amplification, the HotStarTaq® Master Mix Kit supplied by Qiagen, Germany (lot number 203446) was used. The PCR mixture was as follow: 1µl of each primer, 5 µl of template DNA, 12.5 µl HotStar Taq® Master Mix and 4.5 sterile high-quality water. The amplification was performed on an Applied Bio System gradient thermal cycler, USA. The amplification profile included 15 min Initial heat activation at 95°C for one cycle, 1 min at 94° C, 1 min at 64°C and 1 min at 72°C for 35 cycles and 5 min at 72°C for one cycle. *C. trachomatis* plasmid DNA (570 bp) products were detected by electrophoresis on 2% agarose gel after staining with ethidium bromide, Figure 1.

Measurements of DNA sperm fragmentation:

The percentage of sperm DNA fragmentation was performed in fresh semen using flow cytometry by a commercial kit supplied by Coulter (Beckman Coulter, Fulterton, CA, USA). The kit's method is based on the fluorescence emission from individual sperm stained with propidium iodide (PI) excited with a 488 nm argon laser, based upon the ability of PI to bind to DNA. Semen samples were diluted with PBS (pH 7.4) to 2 x 10^6 sperm. Of these, 50 µls were incubated with 100 µls lysing reagent for 15 seconds then 2 ml of PI added, mixed and tube acquisition was performed by flow cytometry (Dako Cytomation, Denmark). In principle, the intensity of the fluorescence emission corresponds to DNA content. Flowcytometric analysis displayed a constant and characteristic bimodal nonartifactual DNA pattern confirming existence of two distinct populations. Figure 2 shows the main population, represented by a peak followed by a shoulder which is the marginal population representing a sperm group altered in the nuclear condensation (DNA damage), yielding unstable chromatin, which appears more stainable that was automatically calculated by the flow cytometer after observing 5000 spermatozoa.

Statistical analysis

The data were analyzed and expressed as mean values ± standard deviations (SD) using SPSS version 17 program (SPSS Inc., Chicago, IL, USA). Unpaired t-test was used in comparison of numerical parametric data. Pearson correlation test was applied to analyses correlations between quantitative variables. Statistical significance was set at $P < 0.05$.

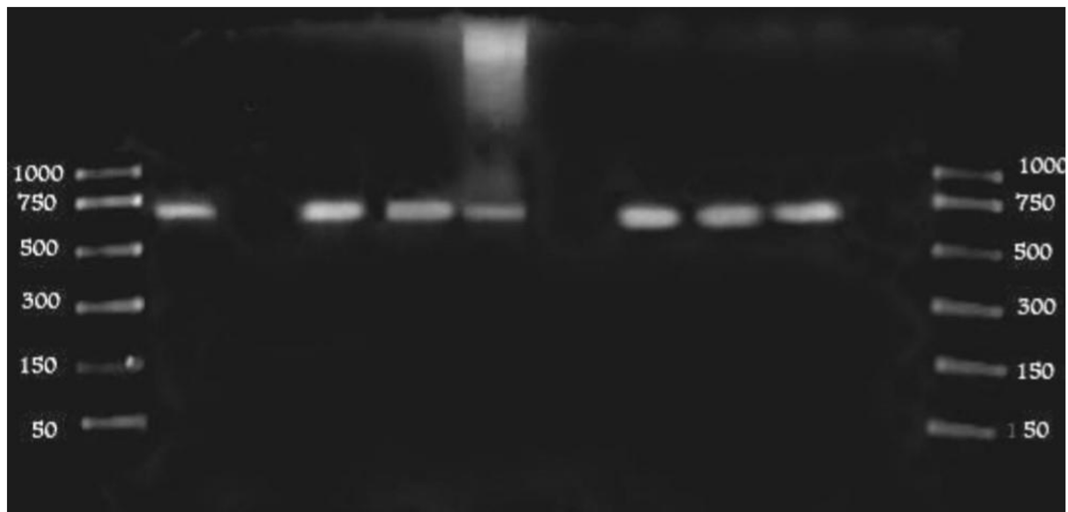

Figure 1. Detection of *C. trachomatis* plasmid DNA by PCR. Positive reactions are detected at 570 bp.

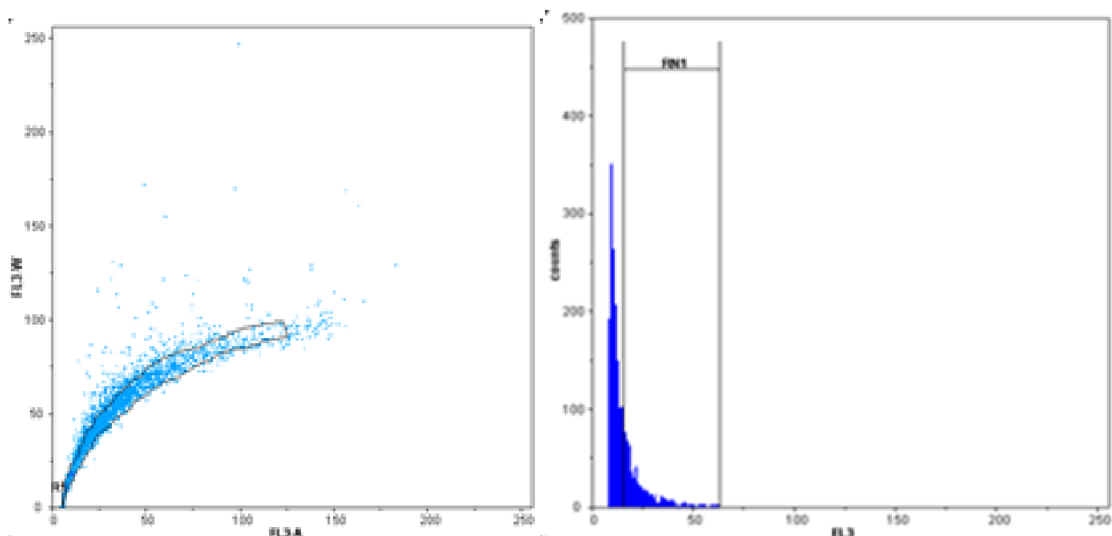

Figure 2. Flow cytometer scatter plot (left) and histogram (right) in the patients and control groups. Spermatozoa cells gated in R1 region in scatter blot were analyzed, while debris and aggregates were excluded from analysis. RN1 region in the histogram represents sperm cells with fragmented DNA. N.B. The percentage of sperm DNA fragmentation in patient with leukocytospermia was 19.7%.

Results

This study was performed on 60 infertile male patients with CT infection. Of these, 35 patients showed leukocytospermia and 25 patients without leukocytospermia. A group of 25 age matched infertile patient with negative chlamydia and no pyospermia served as a control group.

Demographic data, semen analysis assessment, ROS, and the percentage of sperm DNA fragmentation in CT cases (total group), CT cases with leukocytospermia (pus cell $>1x10^6$), CT cases without leukocytospermia (pus cell $<1x10^6$) and the control group are summarized in Table 1. Primary infertility was detected in 43 (68.3%) patients and secondary infertility detected in 17 (28.3%) patients. Semen volume, sperm concentration, and sperm motility were significantly lower in the patient group when compared to the control group ($p < 0.05$). However, no significant difference was observed between CT patients with leukocytospermia when compared to those without leukocytospermia.

Table 1. Demographic data and semen analysis assessment, ROS, and the percentage of sperm DNA fragmentation of patients with CT, patients with leukocytospermia, patients without leukocytospermia, and the control group.

Variables	*Chlamydia trachomatis* patients (n=60)	*Chlamydia trachomatis* patients		Controls (n=25)
		With leukocytospermia <1x106 (n=35)	Without leukocytospermia >1x106 (n=25)	
Age in years				
Mean ± SD	35.30 ± 5.90	35.61 ± 5.9	33.84± 4.3	36.20± 5.74
Median	33.00		33	35.50
Fertility status				
Primary Infertility	43 (68.3%)			-
Secondary Infertility	17 (28.3%)			-
Semen volume (ml)				
Mean ± SD	2.67 ± 1.30	2.17 ± 1.35	2.88 ± 0.96	2.76 ± 0.84
Median	2	-	3	3
Sperm concentration (mil/ml)				
Mean± SD	26.9± 33.13[a]	25.95± 27.05[c]	20.15+ 18.13[d]	80.9 ±47.19
Median	15	15	18.13	67.5
Normal sperm morphology %				
Mean ± SD	37.58±19.27[a]	37.55±20.51	36.13±13.48	57.04±11.1
Median	33	33	33	35
Progressive sperm motility %				
Mean ± SD	34.0±10.59[a]	31.52±10.8[c]9	28.538±4.8[d]5	50.19±11.05
Median	32.50	33.5	26	53
Total pus cell (1x10^{6}/ml)				
Mean ± SD	4.46±4.26[a]	5.1[c,b]±9.23	0.68±0.23[d]	0.68±0.23
Median	2.55	10.1	0.66	0.66
Semen ROS level (RLU)				
Mean ±SD	2267.82±1973.12[a]	2792.66±2057.07[c,b]	861.53±677.46[d]	490.26±449.66
Median	1325	1126.5	671	323
Sperm DNA fragmentation%				
Mean ±SD	16.16±6.00 a	17.068± 4.8 [b,c]	12.17±2.7	8.63±3.07
Median	15.45	17.05	11.7 [d]	8.63

RLU, relative light unit; HOS, hypo-osmotic swelling test; ROS, reactive oxygen species. a: Significant difference between the total patient group and the control group (P<0.05). b: Significant difference between patients with leukocytospermia and those without leukocytospermia (P<0.05). c: Significant difference between patients with leukocytospermia and the control group (P<0.05). d: Significant difference between patients without leukocytospermia and the control group (P<0.05).
P<0.05 is significant.

There was a significant increase in semen ROS levels and the percentage of sperm DNA fragmentation in the CT patient group when compared to the control group and in those with leukocytospermia when compared to those without leukocytospermia ($P < 0.05$).

In the CT patient group, there was a positive correlation between the percentage of DNA fragmentation, ROS (r = 0.82 with $P < 0.0001$) Figure 3, and pus cell count (r= 0.7 with $P < 0.0001$) Figure 4. In patients with leukocytospermia there was a positive correlation between the percentage of DNA fragmentation, ROS (r = 0.7 with $P < 0.0001$) Figure 5, and pus cell count (r = 0.5 with $P < 0.0001$) Figure 6.

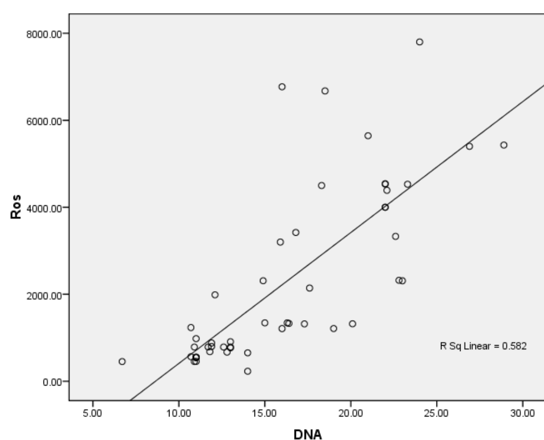

Figure 3. Correlation between ROS and percentage of sperm DNA fragmentation in CT patients. There was a positive correlation between the percentage of DNA fragmentation and ROS (r = 0.82, P = 0.000).

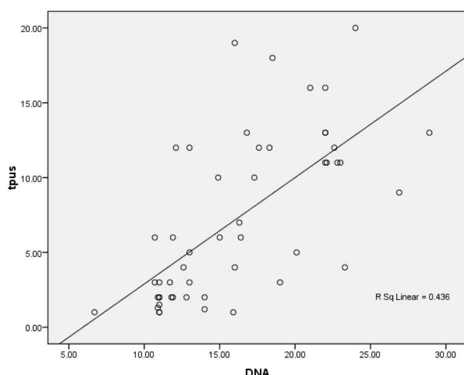

Figure 4. Correlation between semen pus cell count and percentage of sperm DNA fragmentation in CT patients. There was a positive correlation between the percentage of DNA fragmentation and semen pus cell count (r = 0.7, P = 0.000).

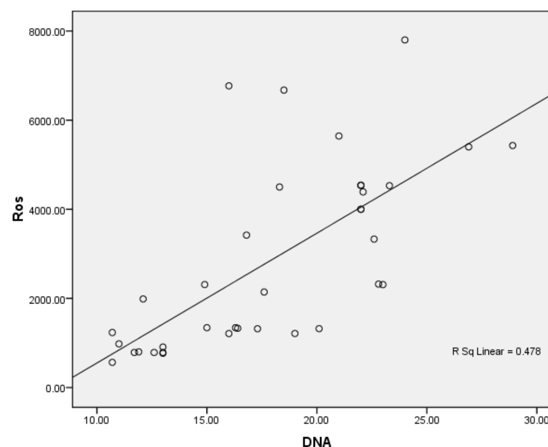

Figure 5. Correlation between ROS and percentage of sperm DNA fragmentation in CT patients with leukocytospermia. There was a positive correlation between the percentage of DNA fragmentation and ROS (r = 0.7, P = 0.000).

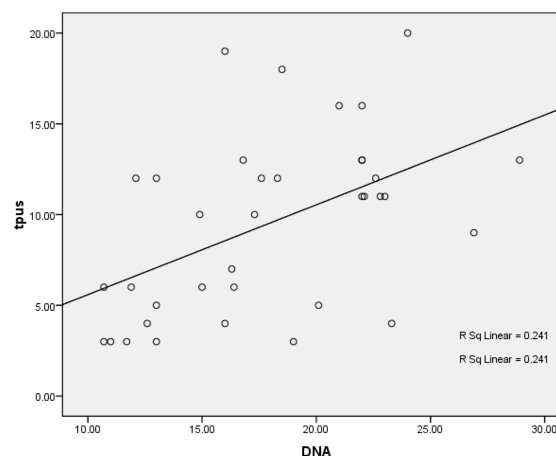

Figure 6. Correlation between semen pus cell count and percentage of sperm DNA fragmentation in CT patients with leukocytospermia. There was a positive correlation between the percentage of DNA fragmentation, semen pus cell count (r = 0.5, P = 0.000).

Discussion

Chlamydia trachomatis infection could affect semen quality through several mechanisms, apoptosis of spermatozoa, persistent infection resulting in scarring of the ejaculatory ducts and damage in the epithelial cells involved in spermatogenesis, can also impair sperm quality. It is considered one of the most prevalent sexually transmitted diseases in the world.

In the present study the prevalence of CT infection (24%) is comparable with other similar studies of Gonzales et al., 2004[11] who found it 26% in USA. Osazuwa et al., 2013.[12] also reported that 19.5 of the infertile males were found to be sero-positive for *C. trachomatis*. Lower results were reported by Moazenchi et al., 2018,[3] found that the CT prevalence was 14.3%, 11% among those with semen abnormalities and 26% among those without semen abnormalities patients. On the other hand, Gdoura et al., 2001[13] found that the prevalence of *C. trachomatis* infections was 35.9% in men and 38.0% in women.

As regarding sperm concentration, mobility, and viability, all such parameters were significantly decreased in patients with CT compared to the control group. These findings are consistent with those of Pajovic et al., 2013[14] and Suarez et al., 2017[15] who reported that the sperm damage due to direct effect of *C. trachomatis* elementary bodies EBs on spermatozoa which lead to premature sperm death through an apoptosis mechanism induced by *C. trachomatis* lipopolysaccharide (LPS) or its soluble products, or indirectly through the induction of tissue inflammation and the release of toxic inflammatory mediators such as ROS and cytokines. Moreover, Suarez et al., 2017[15] also proposed that chlamydial lipo-poly saccharide (LPS), through its binding to CD14 on the sperm surface, could trigger ROS production and caspase-mediated apoptosis. Belloc et al., 2014[16] have shown that *C. trachomatis* can adversely affect sperm function, reducing concentration, quality, velocity, morphology, motility, and viability, altering semen pH and reducing the volume of the ejaculate. In addition, Belloc et al., 2014[16] also found that some infertility or subfertility etiologies can result in dysfunctional spermatogenesis and reduced sperm count due to extrinsic factors include increase ROS which can result in seminal abnormalities that include low sperm count (oligozoospermia), abnormal morphology (teratozoospermia), and reduced motility (asthenozoospermia).

Contrary to our findings, Dehghan et al., 2016[17] failed to find any correlation between *C. trachomatis* infection and semen alternations and development of abnormal semen characteristics among infertile males. Fraczek & Kurpisz 2015[18] also demonstrated no association between *C. trachomatis* infection of the male genital tract and altered sperm quality.

Leukocytospermia has a negative effect on semen parameters, which could be through the production of ROS. In the present study, semen leucocytic count, ROS levels and the percentage of sperm DNA fragmentation significantly increased in CT patients group compared to the control group. Moreover, ROS levels and the percentage of sperm DNA fragmentation were significantly increased in patients with leukocytospermia compared to those without leukocytospermia. Also, the percentage of DNA fragmentation was positively correlated with ROS levels and total pus count, but negatively correlated with motility and sperm concentration. Such findings are consistent with those of Fraczek & Kurpisz, 2015[18] who reported that excessive production of ROS in leucocytes, which has been induced by *C. trachomatis* lipopolysaccharide, may affect semen parameters and DNA integrity.

Dehghan Marvast et al., 2018,[1] also reported that the direct effect of CT elementary bodies (EBs) attachment to sperm lead to premature sperm death through an apoptosis mechanism. Moreover, the cumulative effect (via ROS damage, energy depletion) lead to reducing the sperm survival. These findings are also supported with those of Hussen et al., 2018.[19] Furthermore, Ahmadi et al., 2016[20] reported that patients with genitourinary infection with *C. trachomatis* showed increased sperm DNA fragmentation in comparison with fertile controls. This increase is proportionally greater than the influence on classical semen parameters and could result in a decreased fertility potential. Elevated levels of sperm DNA fragmentation are related to morphological abnormalities,[21] and it is believed that normal sperm morphology can be a valuable predictor of the fertilization rate if it is evaluated by strict criteria.[22] The importance of studying sperm DNA integrity has been further intensified by the growing concern about the transmission of genetic disease through intracytoplasmic sperm injection (ICSI).[23] Based on our study findings,

we may concluded that sperm concentration, mobility and viability, are significantly decreased in patients with CT compared to the control. ROS levels and the percentage of sperm DNA fragmentation significantly increased in CT patients especially patients with leukocytospermia.

Author Contributions

HDG; designed the idea and the tools of the study. AME; revised step by step the recruitment of patients, the tools used, collected, analyzed, interpreted the data and proposed the methodology of the study. DTK made the statistical analysis and interpreted the results and wrote the manuscript. All authors agreed with the results and conclusions of this article. All authors read and approved the final manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The protocol of the study was reviewed and approved by the Ethical Committee of the Faculty of Medicine, Assiut University (approval date: July 2021).

Informed consent

An informed consent was obtained from all patients and controls.

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