

Effect and Mechanisms of Reproductive Tract Infection on Oxidative Stress Parameters, Sperm Dna Fragmentation, and Semen Quality in Infertile Males

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Research

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Effect and mechanisms of reproductive tract infection on oxidative stress parameters, sperm DNA fragmentation, and semen quality in infertile males

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Abstract:

Recent years have seen a rise incidence of male infertility, and mostly caused by the decline of sperm quality. The ratio of infertile males to infertile females has escalated from 3:7 in 2013 to today's 5:5, which turning male infertility into the research focus of reproductive medicine. This study aimed to clarify the effect of reproductive tract infection by ureaplasma urealyticum (UU) and chlamydia trachomatis (CT) on the DNA integrity and routine semen parameters of infertile males. A retrospective study was performed. A total of 259 infertile males who were treated at the Andrological Laboratory Examination and Reproductive Medicine Center in our hospital were analyzed. qRT-PCR was used to examine the infection status of CT and UU. According to the

eligibility criteria, we evaluated the semen parameters and biochemical data of 253 men. Based on the results of PCR, the subjects were divided into four groups: group I (CT positive, 63 cases), group II (UU positive, 60 cases), group III (CT positive and UU positive, 62 cases), and group IV (no infection, 68 cases). DNA fragmentation index (DFI), sperm count, vitality and morphology, elastinase level, seminal plasma malondialdehyde (MDA), and total antioxidant capacity (TAC) were assessed. Compared to group IV, three groups (group I, group II and group III) showed difference in semen volume, proportion of sperm with normal morphology, sperm motility, progressive motility, and vitality ($P<0.05$). Compared to group IV, group II and group III showed difference in DFI ($P<0.05$). Compared to group IV, group II and group III showed difference in elastase level ($P<0.05$). VCL, VSL, VAP, WOB,ROS,TM, HDS showed differences between groups of abnormal/normal WBC ($*P<0.01$)

UU infection significantly increased the level of seminal leukocytes only in group II, but not in the other three groups, indicating that UU is a factor to increase the level of seminal leukocytes. Compared with the normal leukocyte group, there were significant differences in total motility, forward motility and normal sperm ratio between the two groups. The proportion of sperm with abnormal morphology (mostly in the head) showed obvious difference between groups of high and normal seminal leukocytic level. At the same time, in this study, SCGE and SCD verified that leukocytes could damage sperm DNA by increasing ROS, which ultimately affects male fertility.

Keywords: DNA fragmentation test, ureaplasma urealyticum, chlamydia trachomatis, semen parameters, malondialdehyde, total antioxidant capacity; single-cell gel electrophoresis; SCD test

Introduction

Human sperm quality can be affected by various factors, including physical injury, psychological stress, or environmental pollutants[1,2]. Reproductive tract infection, usually presented by non-gonococcal urethritis, is often caused by ureaplasma urealyticum (UU) and chlamydia trachomatis (CT) [3]. Male reproductive tract infection, a big threat to reproductive health, is responsible for the infertility in about 15% patients[4]. Gonococcal urethritis caused by these two types of organisms is more likely to invade male's genital tract, epididymides, prostate gland, and testes, exerting bad effect on semen quality, spermatogenesis, sperm-egg binding, and pregnancy

outcomes[5]. Mycoplasma infection and other causes of chronic prostatitis may also damage sperm DNA[6]. UU, a pathogenic microorganism, can adhere to genitourinary tract and evoke urinary tract infections. It can also affect male fertility in several ways, such as interfering with spermatogenesis, distorting sperm metabolism and sperm-egg binding, as well as triggering immune responses in the genital tract[7].

The methods to detect the two organisms are in continuous renewal. Samples detected with the RNA analytical method far outnumber those with the DNA analytical method, for the former is more specific, precise and time-saving[8]. PCR analysis has a high specificity up to 99-100%, but its false negative results may result from the “carry over” and false positive results from Taq inhibitors in the samples[9]. Multiple PCR shows a sensitivity and specificity close to 100% in UU detection[10]. Nucleic acid hybridization test has a sensitivity of 70-92% and a specificity of 97-98% in detecting CT specific RNA[11]. CT DNA can also be detected by PCR [12]. Nucleic acid amplification test can detect the sequences of CT DNA or RNA, with a sensitivity > 90% and a specificity > 99%. This test has also been approved by FDA to screen and diagnose CT infection[13]. Besides, sperm DNA fragmentation analysis has been developed to evaluate sperm quality and fertility. This method is more accurate than the routine semen analysis the efficiency of which is affected by multiple factors [14]. Human sperm DNA is the carrier of hereditary information of humans. Sperm DNA damage is partly associated with male infertility [15]. The normal reproductive processes, including sperm-egg fusion, membrane fusion, chromosome combination, and embryogenesis, can only be accomplished by sperm with intact DNA[16]. Up to now, few researches use DFI to examine the effect of two organisms on male infertility in China. Therefore, this study was designed to explore the effects of reproductive tract infection on oxidative stress parameters, sperm DNA fragmentation, and semen quality in infertile males.

Materials and methods

Study population

According to the definition of male infertility set by the WHO (Definition of infertility: Failing to fertilize the wife after more than twelve months' cohabitation and normal sex without any

contraceptive intervention (5th ed. Beijing: People's Health Press)[17], a total of 259 infertile males (ages from 18 to 35 years, mean age 31 ± 2.3 years) who were treated at the Andrological Laboratory Examination and Reproductive Medicine Center at our hospital in 2016 and 2017 were included in the present study. Excluded were those with trauma, family diseases, urological and reproductive diseases (like varicocele, cryptorchidism, prostatitis, epididymitis)sexual dysfunction, medication history (hormones or cytotoxic drugs) and occupational exposure (to zinc, high-density radiation, chemicals, high temperature).

Varicocele was diagnosed with ultrasound covering the vessels of pampiniform plexus; no conceiving was observed after > 2 years of regular sexual intercourse (causes from the woman were excluded); ultrasound showed dilated unilateral or bilateral spermatic vein of >2 mm in diameter; valsalva maneuver reduced venous return; palpation showed obvious thickening in spermatic vein with/without ipsilateral testicular atrophy; normal semen parameters or sperm quality [18].

Three semen samples were excluded for their nonconformity to the inclusion criteria (one for medication history and two for missed biochemical data). Next, we evaluated the semen parameters and biochemical data of 253 males. The baseline characteristics are presented in Figure 1. According to the results of CT-DNA and UU-DNA tests, all the patients were divided into four groups: group I (CT positive, 63 cases), group II (UU positive, 60 cases), group III (CT positive and UU positive, 62 cases), and control group IV (no infection, 68 cases). Patients in the four groups showed no difference in age and the duration of infertility.

Ethics

The research was approved by the Ethics Committee of Women's Hospital of Nanjing Medical University, and was conducted in accordance with the Declaration of Helsinki. An information sheet was provided to all participants. Written informed consent was obtained from all participants. The relevant guidelines and regulations of the local institute were strictly followed when conducting the study. Participants were informed that they could withdraw from the trial without giving a reason.

Sample collection

Semen collection

Semen samples were collected by masturbation after 2 to 5 days of ejaculatory abstinence (WHO, 2010)[19]. The duration of abstinence was recorded. Each semen sample was directed into a sterile plastic cup, liquefied in an incubator at 37 °C. After the semen was completely liquefied, at least 2.6 ml of semen was taken for the test (1.5 ml of the semen was used for routine semen analysis, the left for DNA fragmentation analysis and oxidative stress parameters). Semen in the sterile tube was also used for CT-DNA and UU-DNA tests.

Routine semen analysis

According to Laboratory Manual of the WHO for the Examination and Processing of Human Semen (5th edition) and WHO Manual for the Standardized Investigation, Diagnosis and Management of the Infertile Male[19], the routine semen analysis was performed with a semen quality detection system (WLJY-9000, Beijing Weili New century Science & Tech Dev .Co.Ltd) and supporting reagents. Main parameters were as follows[20]. Image acquisition frame: low and middle sperm concentration collected at 20 Hz, and high sperm concentration at 7 Hz; acquisition interval: 3 ms; maximum sperm motile velocity: 200 $\mu\text{m s}^{-1}$; area range of spermatozoa head detected at 7-60 μm^2 . Index of sperm motility: straight line velocity (VSL). Grayscale thresholds were set to collect spermatozoa and exclude nonsperm granules. According to the thresholds set for sperm analysis, sperm images were collected and analyzed.

Sperm morphology assessment

For morphological evaluations, seminal smears were stained with Diff-Quik (MICROPTIC S.L. Co., Barcelona, Spain)²¹. Approximately 10 μl of sperm was smeared into a thin and homogeneous layer on a clean glass slide and was air-dried at room temperature for at least 10 min. The slides were stained and observed under a brightfield microscope (BH-2; Olympus, Tokyo, Japan) at 1000 \times magnification. According to WHO guidelines, a sperm with deformed head, or midpiece, or principal piece was counted as. SDI (sperm deformity index)=number of deformed sperm/number of total sperm²². For each semen sample, at least 200 sperms (or the whole sperm if the slide had less than 200 sperm) were counted via a double-blinded method.

Then, the percentage of sperm with normal morphology was calculated.

DFI(SCD test)

Using the SpermFunc™ DNAf kit (BRED Life Science, Shenzhen, China), SCD test was performed to measure the DNA fragmentation in native and DGC-separated semen. Gelled aliquots of low-melting-point agarose in the kit were provided for semen sample processing in Eppendorf tubes. Eppendorf tubes were placed in a water bath at 80 °C for 20 min to melt the agarose and then transferred to a water bath at 37 °C for 5 min for temperature equilibration. A total of 60 µL of sampled semen was added to and mixed with the agarose in the Eppendorf tubes. Then, 30 µL of semen-agarose mixture was pipetted onto precoated slides in the kit that were covered with a 22 × 22-mm coverslip. The slides were placed on a cold plate in the refrigerator (4 °C) for 5 min, allowing the agarose to produce microgel in which the sperm cells were embedded. The coverslips were gently removed, and the slides were immediately immersed horizontally in solution A and incubated for 7 min. Next, the slides were horizontally immersed in solution B for 25 min. After being washed for 5 min in a tray with abundant distilled water, the slides were dehydrated in gradient concentrations of ethanol (70%, 90%, 100 %; respectively) for 2 min, air-dried, and stored at room temperature in opaque closed boxes.

For bright-field microscopy, the slides were horizontally covered with a mixture of Wright's staining solution (BRED Life Science, Shenzhen, China) and phosphate buffer solution (BRED Life Science, Shenzhen, China) (1:2) for 15 min with continuous airflow. Then, the slides were washed in running water for 10 s and allowed to dry. Strong staining was recommended to allow the periphery of the dispersed DNA loop halos more visible. A minimum of 500 sperm were counted on each sample under the 100 × magnification [23].

Normal spermatid DNA presented radiate halos and damaged spermatid DNA presented no or small halos. Fragmented sperm referred to those having a small or no halo. The thickness of the halo on one side was less than the 1/3 diameter of the head's thinnest part [15]. The rate of sperm DNA fragmentation (%) = $\frac{\text{the number of sperm with fragmented DNA}}{\text{the total number of sperm}} \times 100\%$, and $< 25\%$ was considered normal.

Determination of sperm DNA damage by SCGE(single-cell gel electrophoresis)

The semen samples were centrifuged at 800 g at room temperature for 5 min, and resuscitated in PBS. The cell concentration was adjusted to 2×10^6 /mL. The 85 μ L 1 % agar-agar with normal melting point (excluding Ca^{2+} , Mg^{2+} , 50 °C) was laid on a completely frosted glass slide, covered with cover glass, and placed in a refrigerator at 4 °C for 10 min until solidified. After removal of the cover glass, 10 μ L sperm suspension was mixed with 75 μ L agarose (0.5%, low melting point) at 37 °C, spread on the slide as the second layer, covered with the glass and refrigerated at 4 °C for 10 min until solidified. Next, after removing the cover glass, 75 μ L agarose (0.5%, low melting point, 37 °C) was spread as the third layer, and refrigerated at 4 °C for 10 min until set. Then, cover glass was removed again and the slides were immersed in 4°C precooled lysate (2.5 mol/L NaCl, 100 mmol/L Na_2EDTA , 10 mmol/L Tris buffer, 1% sodium dodecyl sarcosine, pH=10, and the final concentration before use was 1% Triton x-100 and 10% DMSO) for one hour. The slides were removed from the lysis solution and treated with 10 mmol/L dithioethanol (DTT) at 4 °C for 30min, followed by 4 mmol/L of lithium diiodine salicylate (LIS) at 20 °C for 90min. After rinsing with the electrophoretic solution, the slides were placed in the horizontal electrophoresis tank. The electrophoresis tank was filled with freshly prepared pre-cooled electrophoretic solution (1 mmol/L Na_2EDTA , 300 mmol/L NaOH), which was over the glass slides (about 0.25cm above the slides), and placed for 20 min to make the DNA unscrew. Electrophoresis was performed at room temperature for 20 min (1 V/cm, 300 mA). After electrophoresis, 0.4 mol/L Tris (pH 7.5) was used for 3 times, 10 min each time. After staining with 40 μ L ethidine bromide (EB, 5 μ g/mL), the slides were immediately observed and photographed under an inverted fluorescence microscope at 20X magnification (laser wavelength 515 - 560 nm, blocking wavelength >590nm). 50 cells were randomly selected from each slide to measure the tail moment (TM, the percentage of tail DNA in the total DNA multiplied by tail length).

Seminal malondialdehyde and total antioxidant capacity measurement

Spectrophotometry was used to test the level of TAC (U/L) and MDA (nmol/mL). MDA levels were determined using the thiobarbituric acid (TBA) method. Semen samples were centrifuged at 4°C for 15 min with a speed of 2000 r/min. The supernatant was mixed with the reagents supplied

in an MDA Assay Kit (Nanjing Jiancheng Bioengineering Corporation, China, A003-2) and incubated at 95°C for 40 min. Having been cooled at room temperature, the mixture was centrifuged at 4000 g for 10 min. The absorbance of the supernatant was measured at 530 nm. All operations were performed according to the manufacturer's instructions. The MDA concentrations were expressed as nmol/mL[24].

Seminal elastase detection was conducted with Microplate Reader (RT-6000) and kits (Huakang Co. Ltd., Shenzhen, China). First, the seminal plasma was separated from the liquefied semen through centrifugation and diluted with a ratio of 1: 51 (10 of UL vs. 500 UL of solution). The ELISA wells were coated with goat anti-elastase antibodies to trap α -1-antitrypsin complex. After being washed, the wells were added with enzyme-labeled antibodies against α -1-antitrypsin. After being incubated and washed and substrate showed observable color, the absorbance was measured. Results: normal (<290 ng/ml), inapparent infection (290~1000 ng/ml), infection (> 1000 ng/ml) [25].

Detection of sperm ROS

Luminol was used as the probe to detect the products. After being washed by PBS, the sperm cell concentration was adjusted to 2×10^7 /mL. Then, 10 μ L luminol dissolved in DMSO (5 mmol/L) was added to the sperm suspension (400 μ L) and the fluorescence values were recorded immediately. Another 5 μ L of luminol was added in 400 μ L PBS solution as blank control. A mixed suspension of 25ml H₂O₂ and 10 μ L luminol was used as positive control. The fluorescence signal was measured using a fluorescence spectrophotometer for 15 min. The result was presented in relative fluorescence unit[26].

CT-DNA and UU-DNA tests

CT-DNA and UU-DNA tests were performed with PCR and kits (Daan Co. Ltd., Guangzhou, China). (qRT-PCR was performed to determine NG-DNA, CT-DNA, and UU-DNA levels. Using kits (DAAN GENE, Guangzhou, China) and cycler Prism 7500 (ABI, USA), CT-DNA and

UU-DNA tests were carried out: 93 °C for 2 min, , 1 cycle; 93 °C for 45 s and 55 °C for 60 s, 10 cycles; 93 °C for 30 s and 55 °C for 45 s, 30 cycles. For each experiment, the standard curve was generated according to the standard in the kit. Negative and blank control groups were set. The quantified data was software-analyzed. The level of pathogen ≥ 500 copy/ml was considered positive, while < 500 copy/ml was referred as negative[26].

Leukocyte concentration

Leukocyte concentration was determined after special peroxidase staining (Shenzhen Huakang, China). For staining, 0.1 mL of semen was added into the centrifuge tube, and the staining solution was added to 1 mL for Mix well, shaken for 2 min, rested at room temperature for 20-30 min, then shaken and mixed well. A wet smear was prepared and observed under a microscope at high magnification. Leukocytes of peroxidase-positive were stained brown, and peroxidase-negative cells were not stained. According to the number of leukocytes, the samples were divided into the semen leukocyte normal group (semen leukocyte $< 1 \times 10^6/L$) and the semen leukocyte abnormal group (semen leukocyte $> 1 \times 10^6/L$) [27].

Statistical analysis

SPSS (19.0) was used for statistics analyzes. Chi-square test was used for comparison between proportions. P value corrected with $a' = a/2(k-1)$ was used to compare the proportions. Comparison between multiple groups was performed using ANOVA, and comparison between two groups with LSD. Data shown as mean \pm standard deviation were analyzed with t test. The homogeneity of variances was determined with Leven s test. Variables with low homogeneity were treated with Mann-Whitney test. $P < 0.05$ was considered statistically significant.

RESULTS

Semen parameters in the CT positive, UU positive, CT and UU positive, and control group

Table1: Comparison of Semen parameters

<i>Semen parameters</i>	<i>I (n=63)</i>	<i>II (n=60)</i>	<i>III (n=62)</i>	<i>IV (n=68)</i>
Proportion of sperms with progressive motility(%)	39.8±10.6 ^a	39.9±13.2 ^a	35.2±11.3 ^{a b}	59.2±6.2
Vitality	61.2±10.3 ^a	62.3±13.6 ^a	51.4±11.4 ^{a b}	81.2±6.3
Motility(%)	46.8±9.1 ^a	46.3±11.2 ^a	41.2±10.5 ^{a b}	72.2±5.0
Volume (ml)	2.6±0.9 ^a	2.8±1.2 ^a	2.7±1.3 ^a	3.8±1.5
Proportion of sperms with normal morphology(%)	3.9±1.8 ^a	2.9±1.6 ^a	3.2±1.7 ^a	4.2±1.7
Sperm count (million per milliliter)	102.5±87.2 ^a	93.1±91.3 ^a	91.6±83.5 ^a	136.6±81.2

Note: Group I (CT positive), Group II (UU positive), Group III (CT positive and UU positive), Group IV (control).

a: Compared with Group IV. $P<0.05$;

b : Compared with Group I, II. $P<0.05$

These parameters showed difference between three groups (I,II,III) and the control group ($P<0.05$). Three parameters(proportion of sperms with progressive motility, vitality and motility) of Group III showed difference from those of Group I and II ($P<0.05$). (**Table 1**)

Note: Mann-Whitney was used for comparison of sperm count.

Sperm DNA fragmentation, seminal elastase level and oxidative stress parameters in the CT- positive, UU-positive, CT-UU- positive, and control group

Table2: Comparison of sperm DNA fragmentation, seminal elastase level and oxidative stress parameters

<i>Parameters</i>	<i>I (n=63)</i>	<i>II (n=60)</i>	<i>III (n=62)</i>	<i>IV (n=68)</i>
DFI (%)	19.1±9.2	26.5±12.3 ^a	30.3±15.6 ^a	15.6±8.9
Elastase (ng /ml)	565.2±366.7	920.6±751.2 ^a	1241.8±1016.5 ^a	256.5±172.6
MDA (nmol/ml)	3.6±0.2 ^b	5.0±0.2 ^b	5.1±0.3 ^b	1.7±0.2
TAC(U/L)	19.1±2.8 ^b	18.5±1.5 ^b	16.0±2.2 ^b	31.3±5.2

Group I (CT positive), Group II (UU positive), Group III (CT positive and UU positive), Group IV (control).

a: Group II and Group III compared with Group IV, $P < 0.05$ (Figure 2, Figure 3).

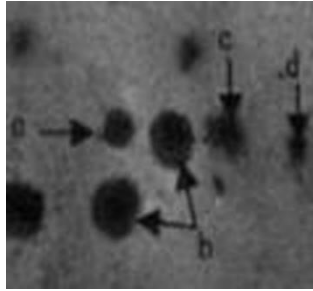
Two parameters (DFI, Elastase) of Group II and Group III showed difference from those of Group IV ($P < 0.05$).

b: Group I, II, III compared with Group IV, $P < 0.05$;

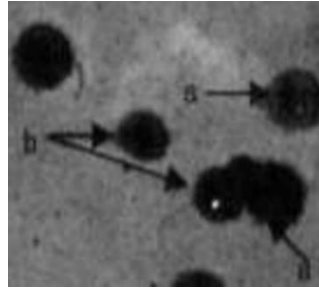
Group I, Group II and Group III showed parameters (MDA, TAC) different from those of Group IV ($P < 0.05$). (Table 2).

DFI: DNA fragmentation index ; malondialdehyde (MDA), total antioxidant capacity (TAC)

A



B



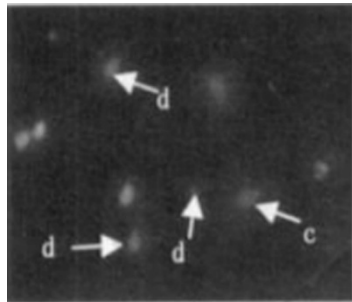
A: Sterile patients with mycoplasma infection; B. Males with normal fertility.

a. Large-size dispersion halos; b. medium-size dispersion halos;

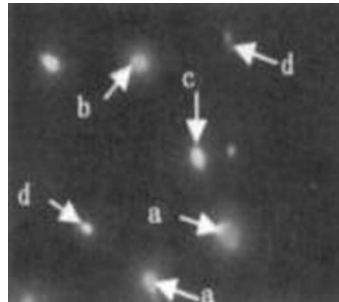
c. small-size dispersion halos; ; d. no dispersion halo

Figure 2 Ordinary light microscope images of sperm in SCD test (Switzer land staining 10×20).

A



B



A: Sterile patients with mycoplasma infection; B. Males with normal fertility.

a.Large-size dispersion halos; b.medium-size dispersion halos;

c.small-size dispersion halos; d.no dispersion halo

Figure3 SCD test, AO staining for fluorescence microscopy(AO staining 10×40).

Proportions of patients with increased seminal leukocytic level in four groups

Table3 : Proportions of patients with increased seminal leukocytic level in four groups

<i>Group</i>	<i>I (n=63)</i>	<i>II (n=60)</i>	<i>III (n=62)</i>	<i>IV (n=68)</i>	<i>X²</i>	<i>P</i>
WBC>10 ⁶ (n=53)	10/63 (15.8%)	20/60 (33.3%)*	17/62 (27.3%)	6/68 (8.8%)	12.6	0.005*
WBC<10 ⁶ (n=200)	53/63 (84.2%)	40/60 (66.7%)	45/62 (72.7%)	62/68 (91.2%)		

Note: Group I (CT positive), Group II (UU positive), Group III (CT-UU-positive), Group IV (control).

Chi-square test was used for comparison. *P* value corrected with a'=a/2(k-1) was used to compare the proportions. *P*<0.05 was considered statistically significant (**P*<0.01) (**Table 3**).

Seminal parameters changed by increased leukocytic level

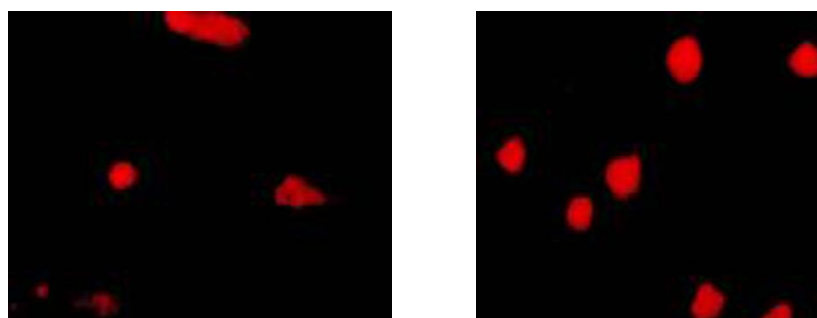
Table 4: Sperm parameters varied as the seminal leukocytic level elevated

<i>Parameters</i>	<i>WBC<10⁶</i> <i>(n=60)</i>	<i>WBC>10⁶</i> <i>(n=53)</i>	<i>t</i>	<i>p</i>
Volume (ml)	2.9±1.1	2.8±0.9	0.2	0.815
PH	7.3±0.2	7.3±0.2	0.8	0.301
Sperm count (x10 ⁶ /ml)	76.2±41.2	75.1±30.2	1.2	0.213
Total motile sperm count, % (x 10 ⁶ /ml)	53.7±25.2	45.3±26.2	2.6	0.008*
Count of sperms with good progressive motility (PR%)	46.2±15.1	38.3±13.4	2.7	0.006*
Proportion of sperms with normal morphology (%)	4.4±1.5	2.9±1.0	1.1	0.005*
VCL (um/s)	46.2±9.0	41.3±9.1	2.2	0.031*
VSL (um/s)	29.5±6.2	26.2±6.1	2.6	0.015*
VAP (um/s)	30.3±6.8	25.0±6.4	2.3	0.018*
ALH (um)	4.0±2.1	4.1±2.1	0.4	0.656
WOB (%)	68.6±5.2	63.3±5.2	3.6	0.002*
Log(ROS+1)	1.8±0.8	2.9±0.6	6.3	0.001*
TM	0.9±0.2	1.2±0.6	3.8	0.009*
HDS(%)	6.5±3.1	11.6±5.1	4.1	0.028*
MDA (nmol/ml)	2.6±0.3	4.5±0.5	0.3	0.035*
TAC(U/L)	23.1±3.3	15.1±1.6	3.5	0.020*
DFI (%)	20.0±10.2	28.2±13.2	2.8	0.022*

Note1: Seminal parameters in groups of abnormal/normal leukocytic levels were compared with t-test. Sixty normal semen samples were randomized as the control. The results showed different total motile count, progressive motility, and proportions of normal-morphology sperms P<0.05 was considered statistically significant (*P<0.01)(Table 4).

Note2: t-test was used for comparison. VCL, VSL, VAP, WOB,ROS,TM, HDS,MDA, TAC showed differences between groups of abnormal/normal WBC (* $P<0.01$).**(Table 4,Figure 4)**

DFI: DNA fragmentation index VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; ALH: amplitude of lateral head displacement; WOB: wobble;TM: tail moment.



I

II

I: effect of abnormal increase of leukocyte on sperm DNA damage;

II : sperm DNA damage in the normal group of leukocyte

The "tail" in SCGE indicates the presence of broken fragments of DNA,Magnification factor ($\times 200$).

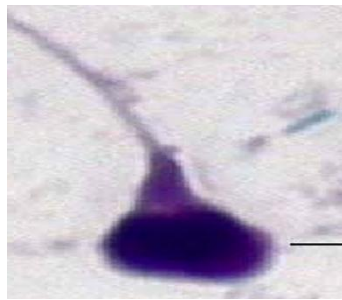
Figure4 Single cell gel electrophoresis image of sperm DNA damage in the group with abnormal increase of leukocyte and the group with normal leukocyte.

Sperm morphology changed by increased leukocytic level

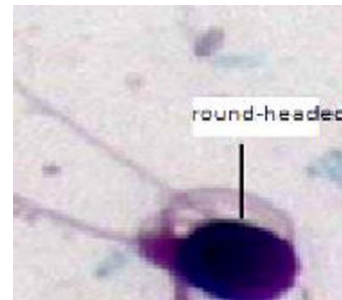
Table 5 The morphology of the sperm changed as the seminal leukocytic level increased

	<i>Proportion of sperms with abnormal morphology</i>	<i>Proportion of sperms with Abnormal head(%)</i>	<i>Proportion of sperms with abnormal mid-piece</i>	<i>Proportion of sperms with abnormal tail %</i>
WBC<10 ⁶ (n=60)	94.6±5.2	75.1±9.9	11.3±3.8	8.3±1.8
WBC>10 ⁶ (n=53)	96.0±3.6	76.2±11.8	12.1±3.6	8.4±2.0
t	2.1	3.1	1.1	0.6
p	0.005*	0.003*	0.511	0.620

Note: Seminal parameters in groups of abnormal/normal leukocytic level were compared with t-test. Sixty normal semen samples were randomized as the control. The results showed that the proportions of sperms with abnormal morphology (mostly in the head) showed difference. $P < 0.05$ was considered statistically significant (Table 5, Figure 5).



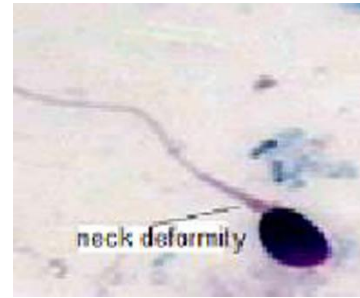
A. Sperm head deformity



B. Sperm head deformity



C: Sperm tail deformity



D: Sperm neck deformity

Figure 5. The photos of sperm deformity. A, B: sperm head deformity; C: Sperm tail deformity; D: Sperm neck deformity.

Discussion

Effects of different reproductive tract infection groups on sperm quality and oxidative stress parameters

Male infertility can be caused by CT and UU infection, and has become to a consensus currently. Most of patients who sufferers from infection-induced infertility present no symptoms [28]. In Asia, the prevalence of reproductive tract infection by mycoplasma is the highest in Iran, followed by India (6.0%), and the lowest in Vietnam (0.8%). In Europe, this prevalence stays at 1.1-7.1%

[29-30], but reaches 17.7% in China and even higher in people who have other STD or do not use condoms during sexual intercourse for more than three months [31]. In our present study, we compared the DFI and other routine seminal parameters in 253 patients. We found that these parameters showed significant difference between the three groups (I, II, III) and the control group, which demonstrated that CT and UU infection can affect seminal quality. In addition, CT and UU damaged DNA integrity and increased the proportion of sperm with fragmented DNA.

Detection of seminal elastase can easily define the infection. If male reproductive tract infection existing, a large amount of elastase will be secreted out of the cell and interact with other oxidants to fulfill its anti-inflammatory role. Elastase, a kind of soluble protease, is evenly distributed in the semen. When used as an index, elastase is more accurate, sensitive and specific than seminal leukocytes in diagnosing male reproductive tract infection, especially inapparent infection [34]. In our research study, elastase levels in group II and group III differed from that in group IV, indicating that seminal vesicles and epididymides can decide the seminal volume and sperm quality. Also, the high-pH environment with an abnormal osmotic pressure increased the death rate of sperm and induced the infection of sex glands.

Male infertility can also be caused by CT-induced reproductive tract infection [35], a process of which inflammatory factors (bacteria, leukocytes) and their metabolites interact to negatively affect sperm genesis and mutation (for example, lowering the sperm density and motility) [36]. CT infection, especially in coexistence with UU infection, can worsen sperm parameters [37]. Thriving in the male reproductive tract, CT produces plenty of phospholipase A₂, breaks arachidonic acid down into prostaglandins, and finally triggers urethritis, epididymitis and orchitis [35]. CT can also penetrate into the sperm, destroy sperm membrane and acrosomal membrane, weaken sperm motility and inhibit the sperm from being fused into egg's pellucid zone. In this process, the fertilization is interrupted and male infertility develops. Like UU, CT can evoke sperm autoimmune reaction. Currently, the mechanisms of CT and UU undermine the sperm quality remain unclear. In the present study, we observed that the sperm motility, progressive motility ability, vitality rate declined obviously in CT-UU-positive group when compared with CT-positive group and UU-positive group (Table 1). Previous studies have not turned to the

synergistic effect of CT/UU infection on sperm quality. In fact, CT and UU act differently in damaging sperm. CT mainly crumbles the sperm structure, but UU mainly binds to hamper the sperm to move. Therefore, our finding may suggest their joint effects. Besides, UU only becomes pathogenic in a given condition. Therefore, once the urinary tract is damaged, sperm environment distorted and sperm structure crumbled by CT infection, UU can accelerate the decline of sperm quality.

Sperm DNA fragmentation is an useful index to assess the sperm quality. Poor sperm DNA fragmentation can reduce males' ability to fertilize, disturb the formation of protokaryotic cells, and even induce fetal abortion, malformation or genetic diseases [38]. CT and UU infection can increase the level of DNA fragmentation. One underlying mechanism may be oxidative stress that can directly damage spermatoblasts or trigger apoptosis. Besides, oxidative stress can change the organization of chromatin, the expression of proteins, and the activity of membrane transporters and receptors involved in ion channels [39]. Some analyses suggest that CT infection and UU infection do not directly decrease sperm count and motility, but increase sperm DNA fragmentation [40, 41]. Our research found that sperm DNA fragmentation in UU-positive group and UU-CT-positive group were statistically different from that in the control group, which is consistent with the results of previous studies [42].

In this study, the control group had an obviously lower seminal MDA level and an obviously higher seminal TAC level than the other groups, indicating that too much MDA was produced during seminal lipid peroxidation and that the drop of TAC level triggered oxidative stress reaction and destroyed the spermatoc membranes [37]. According to Ni et al.[43] and Fu et al.[44], sperm DNA damage could be caused by ROS in patients with varicocele. Shang et al.[45] and Greco et al.[46] have reported that antioxidant can decrease the rate of DNA fragmentation, suggesting that the seminal ROS participates in the process of sperm DNA damage.

Effects of different reproductive tract infection groups on leukocytes

Human semen also contains spermatogenic cells, leukocytes, and genital epithelial cells. According to the definition of WHO, once the level of seminal leukocytes exceeds 1×10^6 mL⁻¹, leukocytospermia arises. Compared with Group IV, the level of seminal leukocytes in Group II elevated significantly, not in the Group I. Bacteriospermia is one cause of infertility. Recent studies have demonstrated the positive correlation between bacteriospermia and leukocytospermia. However, the link between mycoplasma infection and leukocytospermia remains blurry. It is reported that abnormal leukocytic level occurs in 43.4% of seminal samples. Besides, the bacterium-positive rate reaches 48.2% in the group of normal leukocytic level and 54.9% in the group of abnormal leukocytic level (UU being the most frequently detected bacterium). In the present study, we found that UU can increase the level of seminal leukocytes in Group II, which is consistent with the results of previous studies [47]. How seminal leukocytes affect seminal quality is still being debated. As the level of seminal leukocytes increases, the seminal volume, sperm concentration, vitality, and ability to fertilize decrease; in severer cases, the sperm chromatin may be denatured, sperm DNA fragmentation damaged, and the numbers of immature spermatogenic cells and aberrant cells increased. In 2014, Flint et al. reported the association between leukocytospermia and oxidative stress, advocating that ROS plays a dual role: worsening the semen (abnormalizing sperm chromatin and form) and bettering the semen (energizing spermatophores and sperm to survive and move)[48]. Other studies also found that the sperm motility drops as ROS increases in patients with leukocytospermia [49]. These findings may temper the conflicts on leukocytospermia. In the present study, we found that the motility, progressive motility, and the proportion of normal sperms all decreased in the group of abnormal leukocytic level (all $P < 0.05$).

The elevated sperm ROS can lead to sperm tail damage due to cell membrane peroxidation and change the membrane fluidity. At the same time, it can also attack the sperm nuclear DNA and cause DNA damage. Previous studies have found a 1.5 times higher level of oxidized DNA derivatives in infertile men. In order to confirm that the excessive ROS caused by abnormally increased semen leukocytes can attack and damage sperm DNA, we conducted a comparative study on the DNA damage using SCD and single-cell gel electrophoresis(SCGE). The results of SCD revealed that DFI and HDS in the patients with abnormally increased leucocytes were

significantly higher than those with normal leucocyte levels. The results of SCGE showed that the TM value of the abnormal leucocyte group was significantly higher than that of the normal leucocyte group. These results indicate that the abnormal increase of semen leukocytes could cause the fracture and damage of sperm DNA and destroy its integrity and the increased ROS level might be crucial to this mechanism. Sperm DNA compacts the chromatin tightly in the nucleus and stabilizes it in a special way. This characteristic, together with the antioxidants in the spermatoplasm protects sperm DNA from oxidative attack. But because pyrimidines and purine deoxyribose are very sensitive to oxidative stress, excessive ROS can damage sperm DNA. A new research shows sperm DNA fragments are more easily detected in the semen of patients with leukocyte spermatozoosis. And leukocyte spermatozoospermia can induce sperm DNA damage, with cascade amplification effect, while excessive white blood cells are the main source of ROS production in semen[50-51]. In this study, SCGE and SCD verified that leukocytes could damage sperm DNA by increasing ROS. Although the origin and mechanism of sperm DNA damage have not been fully understood, more and more data indicate that DNA damage is closely associated with infertility. Therefore, sperm DNA integrity could be a good indicator of fertility, as a supplement of semen parameters[52]. Excessive ROS caused by increased leukocytes in semen can lead to DNA damage, which is likely to increase the probability of male infertility (Table 4, Figure 4).

The role of leukocytes in the semen has been well explored by the researchers around the world. Here is a to-be-proved mechanism: as the level of seminal leukocytes increases, ROS is plentifully released to ignite peroxidatic reaction in the DHA embedded in mitochondria; as a consequence, less ATP is synthesized than normal, weakening the sperm's ability to accomplish acrosomal reaction and sperm-egg fusion [53]. In addition, the enzymes in leukocytes (peroxidase, elastase, collagenase) and their metabolites (IL-8, IFN-R, TNF) can also damage the sperm, as evidenced by the decreased motility [53]. Recent studies have confirmed that sperm DNA fragments are more easily detected in patients with leukocytospermia and may bring with cascade effects [54].

In the present study, the motile indexes of sperms (VCL, VSL, VAP, WOB) were obviously changed in the group of high-level seminal leukocytes. Studies have verified that the sperm motility decreases along with the leukocytic level increases. In the later process, the

polyunsaturated fat acid (PUFA) undergoes lipid peroxidation, which in turn hardens sperm membrane and drops membrane fluidity, both decreasing the sperm motility. Besides, ROS-induced sperm membrane damage can also increase the permeability, which disables the sperm to regulate the concentration of ions involved in sperm movement; as a result, the motility decreases. Other studies have found that excessive ROS can damage the ultra-microstructure of sperm membrane and mitochondria, which also decreases the sperm motility [55].

High-level seminal leukocytes can also deform the sperms. The proportion of sperms with normal morphology significantly lowers in patients with high level of seminal leukocytes [56]. These findings verify the link between the two indicators. Overproduction of ROS can disturb the sperm-related regulatory mechanism, consequently flawing the sperm structure. In the present study, the morphological indexes showed obvious difference (most in the head) between the groups with abnormal/normal seminal leukocytic level, which is consistent with the previous findings.

Limitations also exist in this research. For instance, the sample size was comparatively small. The effect of sexual abstinence on sperm parameters was not fully considered.

Conclusions

In conclusion, in our present study, we found that the semen parameters showed significant difference between the three groups (I, II, III) and the control group, which indicating that infection of UU and CT in the genital tract can damage seminal quality. Meanwhile, CT and UU infection could damage DNA integrity and increased the proportion of sperm with fragmented DNA. In addition, the proportion of sperm with abnormal morphology (mostly in the head) showed obvious difference between groups of high and normal seminal leukocytic level and the motile indexes of sperms (VCL, VSL, VAP, WOB) obviously changed in the groups of high-level seminal leukocytes, revealing that the sperm motility decreases as leukocytic level increases. At the same time, in this study, SCGE and SCD verified that leukocytes could damage sperm DNA by increasing ROS, which is likely to increase the probability of male infertility. But it's worth noting that the underlying mechanisms of asthenospermia induced by UU or CT remain to be

further explored.

Abbreviations

UU: ureaplasma urealyticum

CT: chlamydia trachomatis

DFI: DNA fragmentation index

MDA: malondialdehyde

TAC: total antioxidant capacity

Ethical Approval and Consent to participate

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the Ethics Committee of Women's Hospital of Nanjing Medical University (No. 20180335) and conducted in accordance with the Declaration of Helsinki (as revised in 2013). All the patients provided the written informed consent.

Availability of supporting data

The datasets used are analyzed are during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

K.S. L, X.D.M wrote the main manuscript, F.P,X.D.Mprepared all the figures, X.Y.Y provided assistance with data acquisition. All authors reviewed the manuscript.

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Figure legend

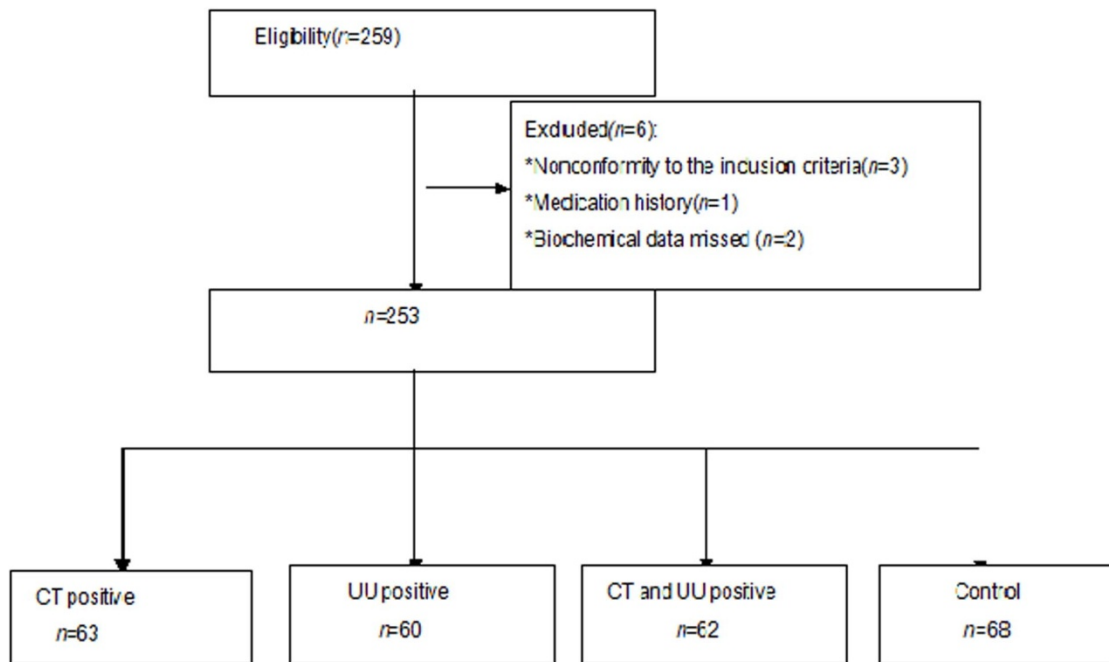


Figure 1: The flowchart of baseline characteristics of participants.

Figure 1: The flowchart of baseline characteristics of participants. After fulfilling the eligibility criteria, we evaluated the semen parameters and biochemical data of 253 men. According to results of PCR, all the subjects were divided into four groups: group I (CT positive, 63 cases), group II (UU positive, 60 cases), group III (CT positive and UU positive, 62 cases), and group IV (no infection, 68 cases). Definitions: CT= chlamydia trachomatis ; UU= ureaplasma urealyticum

Figures

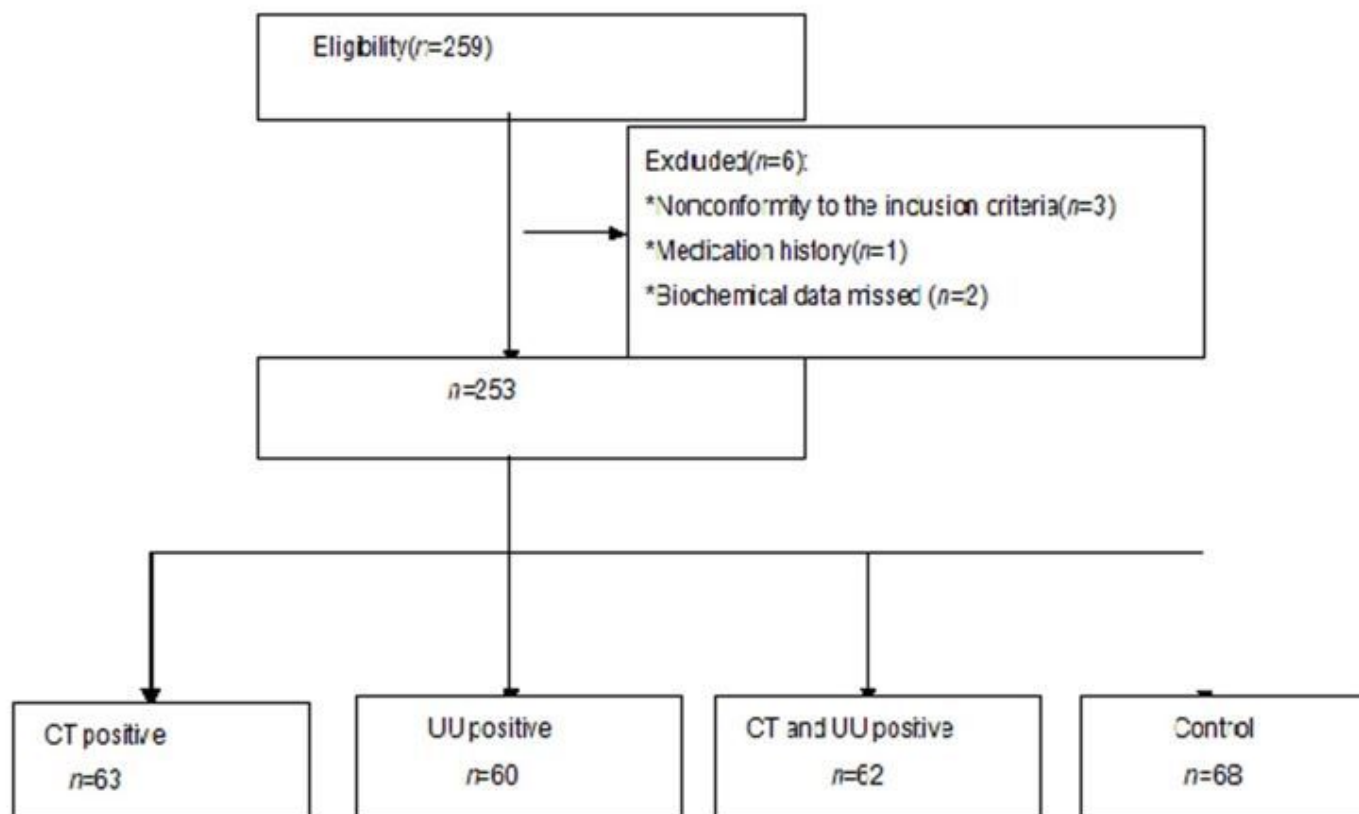
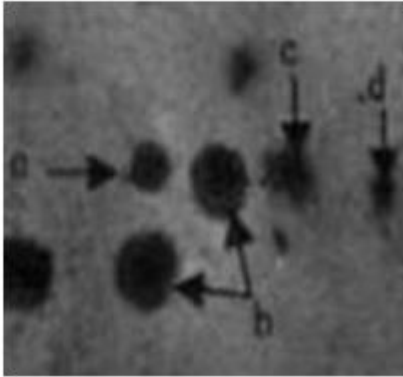


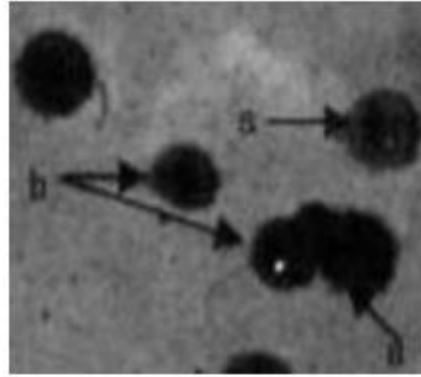
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A



B



A: Sterile patients with mycoplasma infection; B. Males with normal fertility.

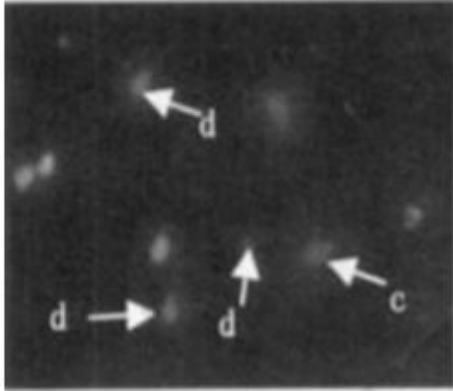
a.Large-size dispersion halos; b.medium-size dispersion halos;

c.small-size dispersion halos; ; d.no dispersion halo

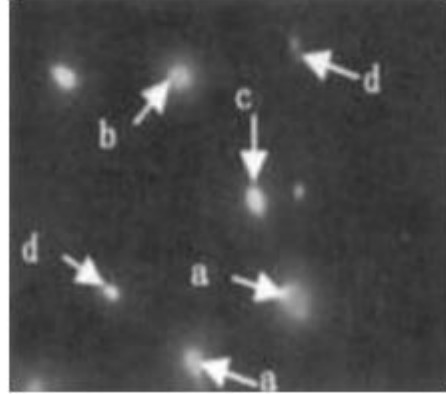
Figure 2

Ordinary light microscope images of sperm in SCD test (Switzerland staining 10×20).

A



B



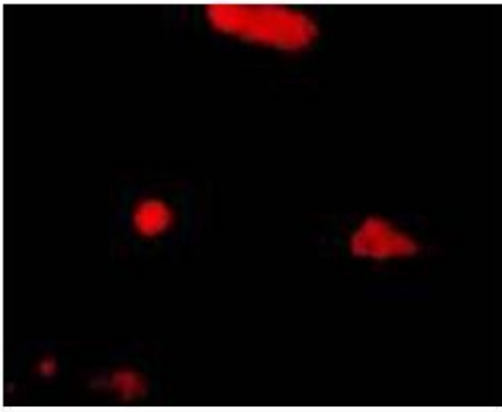
A: Sterile patients with mycoplasma infection; B. Males with normal fertility.

a.Large-size dispersion halos; b.medium-size dispersion halos;

c.small-size dispersion halos; d.no dispersion halo

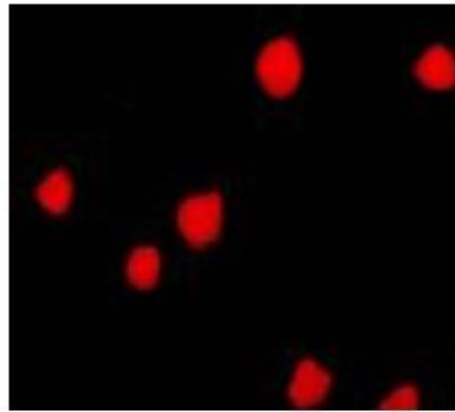
Figure 3

SCD test AO staining for fluorescence microscopy(AO staining 10×40).



I

I: effect of abnormal increase of leukocyte on sperm DNA damage;



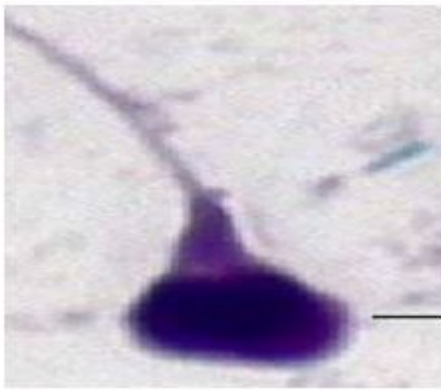
II

II : sperm DNA damage in the normal group of leukocyte

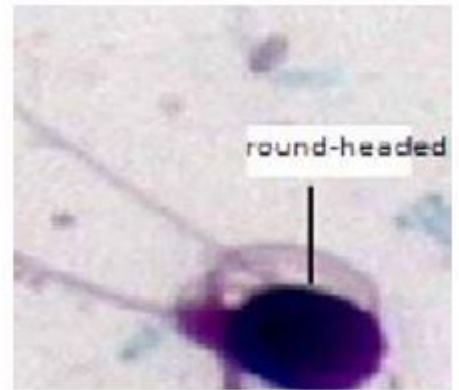
The "tail" in SCGE indicates the presence of broken fragments of DNA, Magnification factor ($\times 200$).

Figure 4

Single cell gel electrophoresis image of sperm DNA damage in the group with abnormal increase of leukocyte and the group with normal leukocyte.



A. Sperm head deformity



B. Sperm head deformity



C: Sperm tail deformity



D: Sperm neck deformity

Figure 5

The photos of sperm deformity. A, B:sperm head deformity; C: Sperm tail deformity; D: Sperm neck deformity.