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Microbiological Evaluation and Sperm DNA Fragmentation in Semen Samples of Patients Undergoing Fertility Investigation

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Abstract: Fifteen percent of male infertility is associated with urogenital infections; several pathogens are able to alter the testicular and accessory glands' microenvironment, resulting in the impairment of biofunctional sperm parameters. The purpose of this study was to assess the influence of urogenital infections on the quality of 53 human semen samples through standard analysis, microbiological evaluation, and molecular characterization of sperm DNA damage. The results showed a significant correlation between infected status and semen volume, sperm concentration, and motility. Moreover, a high risk of fragmented sperm DNA was demonstrated in the altered semen samples. Urogenital infections are often asymptomatic and thus an in-depth evaluation of the seminal sample can allow for both the diagnosis and therapy of infections while providing more indicators for male infertility management.

Keywords: urogenital infections; microbiological evaluation; sperm DNA fragmentation; male infertility

1. Introduction

Nearly 190 million people struggle with infertility worldwide and male infertility accounts for 50% of couples' infertility cases [1]. There are known pre-testicular (attributable to the dysfunction of the hypothalamic-pituitary axis), testicular (mainly testicular pathologies), and post-testicular (urogenital obstructions, vasectomy, and accessory glands impairment) causes that interfere with the composition of seminal fluid and the features of the spermatozoa [2]. In the other cases (30–50%) [1], it is assumed that this

condition is determined by the coexistence of different or harder-to-identify factors (i.e., genetic disorders [3], environmental pollution [4], and infections [5]). In particular, male infertility is defined as unexplained when the parameters of the spermiogram are normal, and as idiopathic when the parameters of the spermiogram are altered without an identifiable cause [1]. The first step of the male fertility routine assessment is semen analysis; this evaluation does not discriminate between fertile or infertile men, but the alteration of some parameters indicates the need for further clinical investigation. Standard semen analysis provides, through a macroscopic (volume, pH, appearance, viscosity, and fluidification) and microscopic (concentration, motility, morphology and presence of non-sperm components) evaluation, data about sperm production and quality [6]. As the semen consists of a concentrated suspension of spermatozoa, stored in the epididymis and, at the time of ejaculation, diluted with the secretions of the accessory glands of the genital tract (mostly prostate and seminal vesicles), some parameters can reflect the sperm capacity of the testicle, the patency of the ejaculatory ducts (the total number of spermatozoa), and the secretory capacity of the accessory glands (the total fluid volume). Both macroscopic and microscopic parameters are considered to highlight inflammatory phenomena [6]. The presence of semen inflammation parameters can be determined by urogenital infections: La Vignera et al. reported an incidence of 13.8% of oligo-astheno-teratozoospermia (OAT) due to the presence of urogenital infections [7]. Infections of the male genitourinary tract account for about 15% of male infertility cases [8]. Infections, acute or chronic, may compromise spermatogenesis and sperm function. A positive semen culture can identify the type and severity of infection by quantifying the colony-forming units. The terms bacteriospermia and infection are distinguished from inflammation since the latter is the response of tissues to an infection [9]. Among infertile men, studies report a prevalence of infections between 11.6% and 45% in cases with a history of urethral discharge as a marker of infection [10,11]. Urogenital infections are also implicated in the pathogenetic mechanisms that alter the nemaspermic cell, such as increasing the percentage of spermatozoa with low mitochondrial membrane potential and apoptosis attributable to the cytotoxic effect exerted by bacteria through membrane permeabilization [12]. To date, a higher percentage of SDF (>30%) was found in infertile subjects compared with fertile subjects (approximately 5–15% SDF) [13–17]. In particular, SDF levels between 30% and 40% are negatively associated with sperm quality and SDF levels of >26% seem implicated in recurrent miscarriage [15–17]. Based on the observations that relate to the integrity of sperm DNA and the outcomes of pregnancy, the SDF evaluation in the diagnostic process of an infertile couple is becoming increasingly important. Therefore, it was proposed as an independent and additional parameter for assessing sperm quality and reproductive potential [14]. The purpose of our study was to evaluate if alterations in human semen parameters (1) are correlated to microbiological agents and (2) sperm DNA damage (Figure 1).

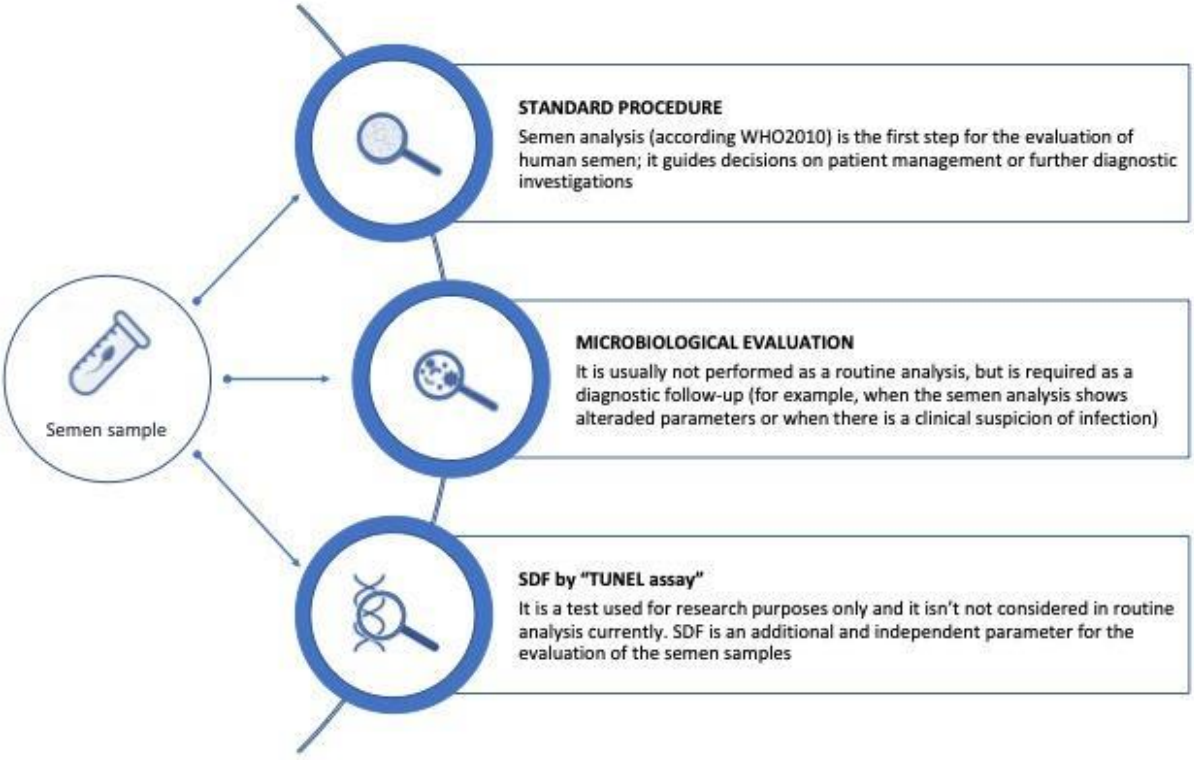

Figure 1. Methods used for the combined assessment of the human semen sample.

2. Material and Methods

2.1. Semen Samples Collection

In this study, 53 semen samples from men (aged 27–44 years) undergoing fertility investigations at the University of Naples Federico II during the period of 1 February 2019 to 31 January 2020 were included. Exclusion criteria for the study included primary gonadal pathologies and genital surgery, a history of radio and chemotherapy, primary or secondary hypogonadism, and concomitant therapies. The investigations were carried out following the rules of the Declaration of Helsinki (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki (accessed on 12 March 2021)). A written informed consent form was signed by all the participants involved in the study (Federico II Ethics Committee, Number: 382-18).

The semen samples were collected between 2 and 7 days of sexual abstinence and the standard semen analysis was carried out according to the WHO protocol of 2010 [6] (Figure 2).

Macroscopic evaluation

Parameter	Ref. limit*	Note	⚠
Liquefaction	15'-60' RT	reflects the activity of prostatic hydrolytic enzymes	the presence of gelatinous clots indicates prostatic inflammation
Appearance	homogeneous, gray-opalescent	reflects changes in the cellular component	opaque, leukocytospermia; aqueous-transparent, sperm reduction; milky, damage of the seminal vesicles; yellowish, pyosmermia
Viscosity	<2cm filament	indirectly correlates with sperm motility	sign of prostatic inflammation
Volume	≧1.5 ml	reflects the secretory activity of the glands and the patency of the ejaculatory ducts	alteration indicates inflammation of seminal vesicles
pH	≧7.2	derives from the balance of secretions from seminal vesicles (alkaline) and prostate (acid)	alkaline ph is a sign of infection or prostatic inflammation; acid pH indicates blockage of seminal vesicles

Microscopic evaluation

Parameter	Ref. limit*	Note	⚠
Total sperm motility (PR +NP)	≧40%	indicates the movements of sperms, both active and in situ	decrease indicates a prostatic inflammation; oxidative stress
Progressive motility (PR)	≧32%	indicates that sperms perform active movements with linearity or in large circles; related to pregnancy rate	decrease indicates a prostatic inflammation; oxidative stress
Sperm concentration	≧15 x 10^6 ml	it is influenced by volume of secretions produced by accessory glands; related to time to pregnancy and pregnancy rate	decrease indicates a prostatic inflammation
Total sperm number	≧39 x10^6 ejaculate	related to time to pregnancy and pregnancy rate	/
Sperm morphology	≧4% (normal forms)	reflects the functional skills of sperm; predictive value for fertilizing potential	increased presence of abnormal forms indicates prostatic inflammation
Aggregation	rare or absent	occur when sperms adhere to cell debris, leukocytes or other elements	
Agglutination	rare or absent	occur when sperm adhere to each other in a site-specific way	suggests an immunological reaction
Non spermatic cells	rare	Epithelial cells derives from genitourinary tract or from efferent ducts (ciliary tufts)	increased presence of erythrocytes indicates prostatic inflammation, obstructions, neoplasia
Round cells	germ cells <10% leukocyte: <1x 10^6 ml	Indicative of testicular damage (germ cells) or inflammation of accesory glands (leukocytes)	leukocytospermia is inflammation sign of urogenital tract

Figure 2. Semen analysis**.** Macroscopic and microscopic parameters used to evaluate the seminal sample. * The reference values refer to the lower 5th centiles (95% confidence interval) regarding the standards of conventional statistics applied to clinical chemistry.

Semen samples were analyzed through macro- and microscopic evaluation using WHO's methods at the time (see Supplemental Material). Measurements were compared with the reference values, taken as reference to the cut-off at the lower 5th percentile, and all the alterations found were recorded.

2.2. Microbiological Evaluation

Before collecting seminal plasma, the patients proceeded with urine collection to better differentiate the infection of the seminal tract from urinary tract infection. About 1 mL of the semen sample was diluted (1:10) with sterile saline solution and centrifuged at 1500 rpm for 15 min at room temperature. After removing the supernatant, the sediment was resuspended in 100 µL of sterile saline solution or sterile saline solution with 10% glycerol. This procedure increases cultural sensitivity because it concentrates bacteria in the cell pellet and eliminates the seminal plasma, which can exert an inhibitory effect on bacterial growth. The cell pellet was spread on different culture media such as Becton Dickinson (BD) Trypticase Soy Agar with 5% sheep blood and McConkey agar for aerobic bacteria, BD Sabouraud Agar for fungi, BD Gardnerella Agar for searching *Gardnerella vaginalis*, and BD Chocolate agar for fastidious bacteria. All media were incubated at 37 °C. To evaluate viable bacteria, BD Trypticase Soy Agar, McConkey agar, and BD Sabouraud Agar were incubated under aerobic conditions for 24 h and 48 h, separately; to allow for the growth of *G. vaginalis* and fastidious bacteria, BD Gardnerella Agar and BD Chocolate Agar were incubated at 37 °C with 5% CO_2 for 48 h. All clinical isolates were definitively identified by MALDI-TOF analysis [18].

Sexually transmitted pathogens, with fastidious growth requirements or non-cultivable characteristics, such as *Ureaplasma urealyticum*/*Ureaplasma. parvum*, *Mycoplasma hominis*/*Mycoplasma genitalium*, and *Trichomonas vaginalis*/*G. vaginalis*, were searched for in the seminal fluid by multiplex real-time PCR. The semen samples were equilibrated at room temperature, mixed by vortexing and 100 µL was pre-treated with a lysis reagent buffer.

The DNA was extracted from the specimens using a RealLine DNA-Express kit (Bioron Diagnostics GmbH, Romerberg, Germany) following the manufacturer's instructions, and stored frozen at −20 °C until testing. The detection of mycoplasmas, ureaplasmas, and *T. vaginalis*/*G. vaginalis* was performed by employing RealLine STI Pathogen Kits (BIORON Diagnostics), multiplex real-time PCR assays for the qualitative detection of sexually transmitted infection (STI) pathogen DNA. The RT-PCR tests were performed according to the manufacturer's protocol. The amplification was performed in a CFX96 Real-Time thermocycler (Bio-Rad, Hercules, CA, USA). Each PCR was performed with 50 µL of extracted DNA. The thermal cycle conditions consisted of an initial incubation at 50 °C for 2 min, pre-denaturation at 95 °C for 2 min, followed by 50 cycles of alternating incubations: denaturation at 94 °C for 10 s, and annealing and extension at 60 °C for 40 s. Samples were considered positive with an average cycle threshold (Ct) value of ≤40, except for *U. parvum* and *G. vaginalis*, which had a Ct value of ≤32. For the detection of *Chlamydia trachomatis*/*Neisseria gonorrhoeae* DNA, the Xpert® CT/NG system (Cepheid, Sunnydale, CA, USA), fully automated real-time PCR test, was used.

The evaluation of the infectious status was standardized, following the instruction of Calogero et al. [19]. In details, based on the etiological agent, each was assigned a score: for sexually transmitted agents (STAs), a score of 3 was assigned; Gram-negative bacteria, a score of 2; Gram-positive bacteria, a score of 1; and for the presence of commensal flora, no score was assigned. The bacterial load was scored as follows: severe bacterial load (>10^4 CFU/mL), a score of 3; moderate bacterial load (10^3–10^4 CFU/mL), a score of 2; mild bacterial load (>/= 10^3 CFU/mL), a score of 1; and reduced bacterial load (<10^3 CFU/mL), a score of 0. The sum of the scores (etiological agent plus bacterial load) determined the infective value for the analyzed semen samples; if the obtained value was greater than 3, the sample was defined infected; otherwise, if the value was equal to or lower than 3, the semen was defined as not infected.

2.3. Sperm DNA Fragmentation Analysis

The sperm DNA fragmentation (SDF) analysis was carried out for each sample by a TUNEL assay using an Apo-Direct kit (BD Pharmingen, CA, USA) through a flow cytometer. The step-by-step approach to the measurement of sperm DNA fragmentation was carried out as follows: an aliquot containing 5 × 10^6/ mL sperm (control and patients) was pipetted into each tube. Once removed, the seminal plasma spermatozoa were fixed in 1.0 mL of paraformaldehyde (1%) in ice for 30 min. Samples were washed in phosphate-buffered saline (PBS) (centrifuge at 300 ×*g* for 7′) and the pellets were resuspended with 1 mL of ice-cold ethanol (70%) in ice for at least 30′. A total of 2 mL of both negative and positive assay controls, provided in the kit, was aliquoted in duplicate. All samples were centrifuged at 300 ×*g* for 7′. The supernatant was carefully removed by aspiration without disturbing the cell pellet. Following this, 1.0 mL of wash buffer was added to each tube, vortexed, and centrifuged twice. The staining solution was prepared according to the manufacturer's instructions. Once prepared, 50 µL of the staining solution was added to all the tubes before being covered with aluminum foil and incubated for 60′ at 37 °C. At the end of the incubation period, 1.0 mL of rinse buffer was added to each tube, and the mixture was centrifuged at 300 ×*g* for 7′. The supernatant was then discarded and this step was repeated. The samples were analyzed by a flow cytometry equipped with a 488 nm argon laser as the light source. Two dyes were used: propidium iodide (PI) for total DNA staining and fluorescein isothiocyanate-2′-deoxyuridine-5-triphosphate (FITC-dUTP) for fragmented DNA staining. Each sample was run in duplicate and cells positive for TUNEL were defined as those containing fragmented DNA. The results are expressed as the percentage of sperm with DNA fragmentation (%SDF) using the flow cytometer software. A minimum of 10,000 events were recorded.

2.3. Statistical Analysis

The parametric T-test on the comparison between means was used to evaluate the statistically significant differences between the semen samples of the Test and Control groups with respect to the study parameters. Pearson's correlation coefficient was used to measure the linear correlation between the sperm parameters and infective status, or %SDF, of the Test and Control groups. A chi^2 test was applied to calculate the %SDF significance between the groups analyzed and the odds ratio was calculated with 95% confidence.

3. Results

The samples were divided into two populations based on the presence or absence of an alteration of the parameters as revealed by the standard semen analysis.

Among the 53 enrolled subjects, 37 were reported to have altered parameters at semen analysis (Test group) and 16 had no semen alteration (Control group).

The microbiological evaluation showed that 70.2% (26/37) of the analyzed semen samples belonging to the Test group presented with multiple microbial agents (values greater than three), as reported in Figure 3. The analysis revealed Gram-negative bacteria in 13 samples, sexually transmitted agents in 10 samples, and the presence or co-presence of Gram-positive bacteria in 24 samples (Figure 3).

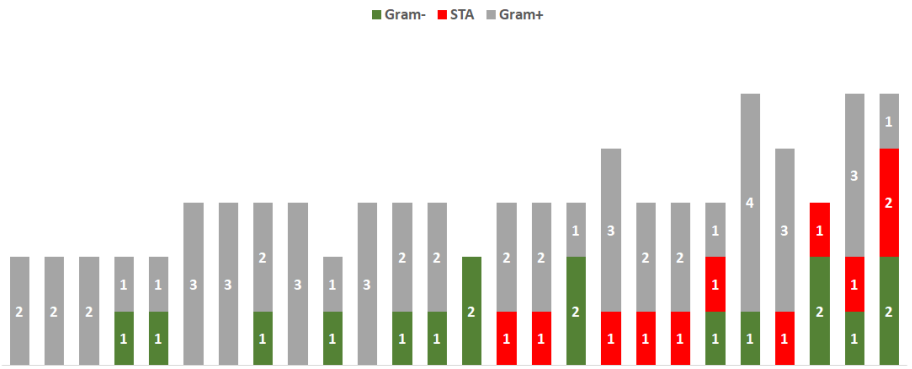

Figure 3. Prevalence of infectious agents in semen samples belonging to the Test group. Each histogram represents a single semen sample: the presence of Gram-positive bacteria was found in 24 samples (in gray), 13 Gram-negative (in green), and 10 sexually transmitted agents (STAs, in red).

In the Control group, just 31.2% (5/16) of the samples presented with an infective value greater than three. The microbiological agents identified in the two analyzed groups are reported in Figure 4.

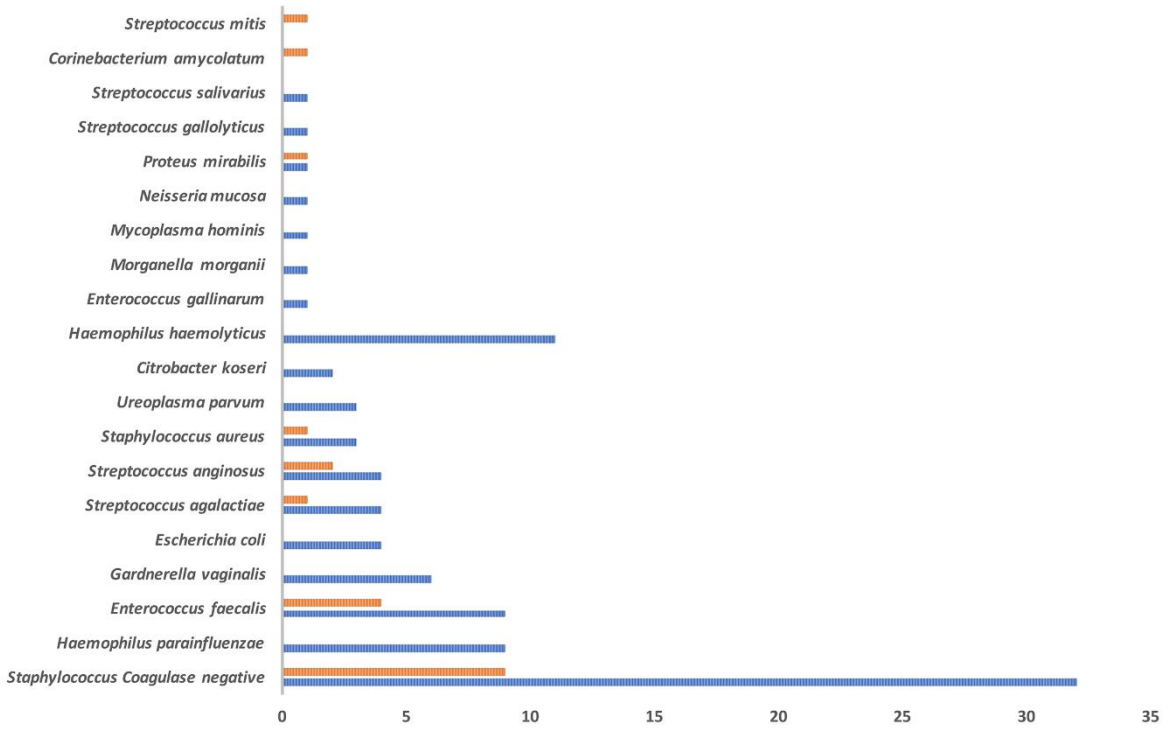

Figure 4. Microbiological evaluation of the collected semen samples among the Test (blue histogram; 37 samples) and the Control (orange histogram; 16 samples) groups. Histograms are used to report the number of samples positive for each microorganism.

Next, in order to highlight if the infective status is related to specific semen alterations, a correlation analysis was carried out. In the Test group, the statistical analysis showed a significant negative correlation between infectious status, semen volume, and total sperm concentration (*p*-value 0.038 and 0.014, respectively) (Table 1). Moreover, a significant correlation was found between the infective status and seminal motility. In detail, the infective status was significantly inversely correlated with specific motility parameters, such as PR + NP% and PR% (*p*-value 0.005 and 0.008, respectively) (Table 1).

Table 1. Correlation between the sperm parameters of the Test group and infective status.

Parameters	Correlation Coefficient	*p*-Value
pH	0.046	0.790
Volume	**−0.346**	**0.038 ***
Conc (×10^6 mL)	−0.223	0.192
Conc/tot	**−0.404**	**0.014 ***
Motility PR + NP (%)	**−0.460**	**0.005 ****
Motility PR (%)	**−0.437**	**0.008 ****
Leucocytes (1 × 10^6/mL)	0.185	0.280
Normal forms (%)	−0.258	0.135

PR: rapid progressive; NP: non-progressive. The statistical significance of the correlation coefficient was evaluated by a T-test student (*p*-value < 0.05, *; *p*-value < 0.01, **).

The same analysis was carried out for the Control group and no correlation was found between the semen parameters and infection status. The sperm DNA fragmentation analysis for the Test group showed that 47.2% (17/36) had an SDF greater than or equal to 30%, 33.3% (12/36) had a %SDF between 15% and 30%, and 19.4% (7/36) had an SDF between 5% and 15%. The same analysis carried out on semen samples from the Control group showed that 12.5% (2/16) had an SDF greater than or equal to 30%, 50% (8/16) had

an SDF between 15% and30%, and 37.5% (6/16) had an SDF between 5% and 15% (Figure 5). Semen samples belonging to the Test group were six times more at risk of a high degree of DNA fragmentation than Control groups with a %SDF level higher than 30% (odds ratio (OR) 5.95, 95% CI, 1.18–29.96) Table 2. Moreover, the semen samples with microbial agents showed a higher percentage of SDF compared with negative samples (Supplementary Figure S1).

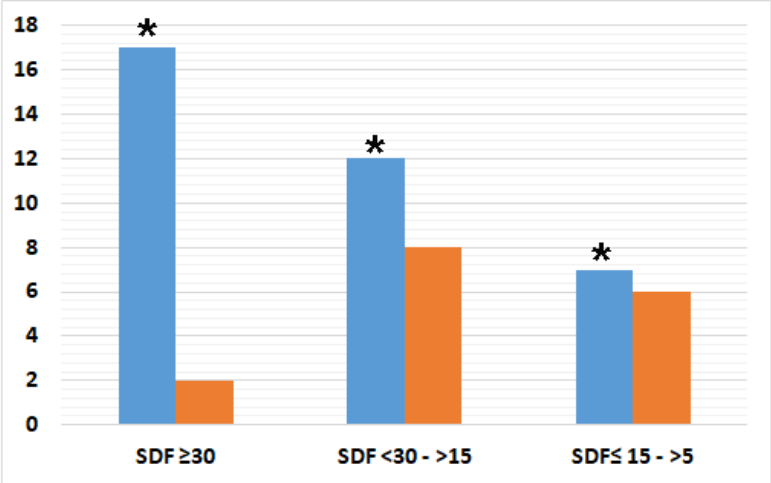

Figure 5. The sperm DNA fragmentation analysis on semen samples within the Test (blue histogram) and Control (orange histogram) groups. The significant difference was calculated through C\the chi² test (*p*-value 0.049).

Table 2. Risk of sperm DNA fragmentation on Test semen samples vs. Control semen samples.

%SDF Levels	Person's Chi Squared	*p*-Value	OR (95%CI)
≥30	0.020	**0.029**	5.95 (1.18–29.96)
<30–≥15	1.926	0.165	0.40 (0.11–1.49)
≤15–>5	1.300	0.254	0.50 (0.15–1.66)

OR: Odd's ratio calculated with a 95% confidential interval.

The correlation between the sperm parameters and SDF is shown in Table 3: in samples of the Test group, a significant negative correlation of pH parameter (*p*-value 0.028), motility PR % (*p*-value 0.036) and motility NP % (*p*-value 0.047) was found. No significant correlation was found between the SDF and the semen parameters of samples belonging to the Control group (Table 3).

Table 3. Correlation between sperm parameters and %SDF in semen samples from the Test and Control groups.

Parameters	Test Group SDF Tunel	*p*-Value	Control Group SDF Tunel	*p-Value*
pH	**−0.362**	**0.028 ***	−0.067	0.806
Volume	0.228	0.174	−0.005	0.987
Conc (×10⁶ mL)	0.031	0.857	−0.277	0.298
Conc/tot	0.114	0.501	0.083	0.761
Motility PR + NP (%)	**−0.336**	**0.042 ***	0.272	0.309
Motility PR (%)	**−0.346**	**0.036 ***	0.322	0.224
Leucocytes (1 × 10⁶/mL)	−0.037	0.827	−0.214	0.426
Normal forms (%)	−0.190	0.268	−0.177	0.512

PR: rapid progressive; NP: non-progressive. Student's *t*-test was used to evaluate the statistical significance of the correlation coefficient (*p*-value < 0.05, *).

4. Discussion

The etiopathogenetic mechanisms that determine potential damage to spermatozoa are numerous and act at different levels: sperm cells can be damaged directly (with evidence of sperm alterations ranging from concentration, motility, morphology to DNA fragmentation, mitochondrial function, apoptosis, and acrosomal reaction) by pathogenic germs [20], bacterial products [21], toxic metabolites produced by microorganisms [21], seminal leukocytes, and soluble factors, such as reactive oxygen species (ROS) and cytokines [22]. In particular, oxidative stress during sperm transport through the male reproductive tract is likely the most frequent cause of sperm DNA damage [23,24].

However, it is not possible to deduce if there is damage of the spermatozoa from the mere presence of leukocytes or other parameters warning of inflammatory status [25–29]. Therefore, in order to achieve the aim of the study, 53 semen samples from subjects undergoing fertility investigation were subjected to standard, microbiological, and sperm DNA fragmentation analysis.

Infection status with a score greater than three was revealed in 70% of semen samples with altered parameters. The results showed that infections deeply affect sperm motility, concentration, and volume. Therefore, dysbiosis in the male reproductive tract microbiota can lead to seminal abnormalities.

Accordingly, in previous studies, bacterial virulence factors detected in human semen samples were reported to deteriorate semen quality by triggering a local inflammatory reaction [30,31].

The correlation between infected status and motility can be explained by the action of cytokines, which are able to modulate and regulate immune and inflammatory responses and to modify the behavior of other cells, inducing new activities such as growth, differentiation, and apoptosis [32]. The molecules most involved in infertility are interleukin (IL) -1, IL-2, IL-6, IL-8, interferon-ɣ, and tumor necrosis factor-α (TNF-α) [33]. TNF-α was shown to reduce sperm motility and increase the percentage of spermatozoa with in vitro early and late apoptosis indices.

The inflammatory response of the genitourinary tract to the invasion of microorganisms is known to activate the release of leukocytes and inflammatory mediators (ROS and cytokines) that affect sperm DNA integrity and negatively influence fertility [34]. Accordingly, in the group of semen with altered parameters and positive infections, a risk six times higher to have a high degree of DNA fragmentation was found. Moreover, considering the presence of microbial agents in semen samples, the %SDF was higher than semen samples without microorganisms.

Leukocytospermia can lead to an overproduction of ROS, which, through the phenomenon of lipid peroxidation of the membranes, alter lipids, proteins, and DNA, thus damaging the sperm membrane and mitochondria, with consequent alterations in motility and sperm DNA [27]. Further sources of ROS in semen are the sperm, particularly immature sperm with cytoplasmic retention and abnormal head morphology characterized by the retention of residual cytoplasm [28]. Both leukocytospermia and the retention of residual cytoplasm within the sperm were associated with increased sperm DNA damage, likely secondary to an increased level of ROS produced by these cells [29].

Since urogenital infections are often asymptomatic, the microbiological investigation is useful for completing the assessment of male infertility in the presence of altered semen sample parameters [35]. Furthermore, in our study, 31.2% of the analyzed samples within the Control population (i.e., with no alteration of semen parameters) had an infective score greater than 3. It is likely the detected infection was of recent acquisition, so no altered seminal parameters were found in association, nor was there an increased percentage of spermatozoa with fragmented DNA equal to or greater than 15% [36].

Sperm DNA integrity was considered an additional predicting factor of male fertility since infertile subjects showed a high percentage of fragmented DNA [15–18].

In particular, results showed that 47.2% of semen samples from the Test group had an SDF greater than 30% vs. the 12.5% of the Control group. According to literature data,

high levels of sperm DNA damage were correlated with poor seminal parameters such as motility [37–39]. However, reports revealed that the standard semen analysis produces normal results in 15% of male factor infertility cases [40].

In addition, discordant results were observed when examining the relationship between fragmented DNA testing and in vitro fertilization (IVF) success rates [41]. A systematic review and meta-analysis in 2016 analyzing 30 studies showed that fragmented DNA testing had limited ability to predict pregnancy in assisted reproductive techniques, especially between IVF and intracytoplasmic sperm injection (ICSI). Other similar articles showed decreased rates of IVF success with higher fragmented DNA [42–44]. An SDF >30%, especially >40%, is often considered a direct cause of reproductive failure, including in IVF and ICSI outcomes. Moreover, a >40% SDF is considered a risk factor of spontaneous abortion [45]. Moreover, since the IVF techniques do not occur in a sterile environment, bacteria can affect semen and embryo quality, resulting in a worse clinical outcome [46].

Considering what was mentioned above, DNA integrity can be an indicator for preimplantation genetic testing [47,48].

The predictive value of sperm DNA fragmentation tests depends on several factors, some related to the damage to the sperm (for example, percentage of damaged sperm, extent of DNA damage per sperm, and combination of DNA fragmentation and nucleotide damage) and others related to the ability of the oocyte to repair damage to the sperm DNA (the oocyte can repair single-stranded damage, while double-stranded damage is irreversible) [49,50]. Despite the known relationship between SDF and semen quality parameters, the lack of a standardized method for evaluating DNA damage in a routine diagnostic setting limits its use in the assessment of fertility. An integrated approach based on standard analysis and microbiological evaluation of semen samples, as proposed in the present study, can suggest an alteration in DNA integrity in patients with suspected infertility.

5. Conclusions

These data underline the importance of a thorough evaluation of couples with unexplained infertility and the need to focus on male factors. In particular, it is necessary to consider semen microbiological status, as these infections are often featured by a paucisymptomatic course. As a consequence, infections affecting the genitourinary tract are often diagnosed too late, after they have already spread to one or more accessory sex glands, thereby becoming chronic and more difficult to eradicate.

The importance of following up on the alterations found in semen analysis, infection status, and DNA fragmentation is evident and can help drive eventual treatments and characterize undiagnosed and unexplained infertility.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Difference of %SDF between semen samples with and without microbial agents.

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Institutional Review Board Statement: The investigations were carried out following the rules of the Declaration of Helsinki of 1975 (https://www.wma.net/what-we-do/medical-ethics/declaration-

of-helsinki, revised in 2013. A written informed consent form was signed by all the participants involved in the study (Federico II Ethics Committee, Number: 382-18).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Abbreviations

OAT	oligo-astheno-teratozoospermia
Ct	cycle threshold
FITC	-dUTP fluorescein isothiocyanate-2′-deoxyuridine-5-triphosphate
IL	interleukin
ICSI	intracytoplasmic sperm injection
IVF	in vitro fertilization
NP	non-progressive
PBS	phosphate-buffered saline
PI	propidium iodide
PR	rapid progressive
ROS	reactive oxygen species
%SDF	percentage of sperm DNA fragmentation
SDF	sperm DNA fragmentation
STA	sexually transmitted agents
STI	sexually transmitted infection
TNF-α	tumor necrosis factor-α

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