Article

Discovery Medicine 2024; 36(184): 959–970
https://doi.org/10.24976/Discov.Med.202436184.89

Impact of *Escherichia coli*, *Candida non-albicans*, and *Trichomonas vaginalis* on Semen Chemical and Functional Parameters: an *In-Vitro* Study

Enas A. El Saftawy[1,2], Rania Y. Shash[3], Heba M. Elwi[4], Ahmed Samir Gamal[5], Mohamed F. Farag[6], Basma Emad Aboulhoda[7,*], Mansour Alghamdi[8,9], Emad A. Albadawi[10], Hadel M. Alghabban[10], Ahmad Motawi[11]

[1]Department of Medical Parasitology, Faculty of Medicine, Cairo University, 12613 Cairo, Egypt
[2]Department of Medical Parasitology, Armed Forces College of Medicine, 4460015 Cairo, Egypt
[3]Medical Microbiology and Immunology Department, Faculty of Medicine, Cairo University, 12613 Cairo, Egypt
[4]Department of Biochemistry, Faculty of Medicine, Cairo University, 12613 Cairo, Egypt
[5]Dermatology, Venereology, and Andrology, Armed Forces College of Medicine, 4460015 Cairo, Egypt
[6]Department of Medical Physiology, Armed Forces College of Medicine, 4460015 Cairo, Egypt
[7]Department of Anatomy and Embryology, Faculty of Medicine, Cairo University, 12613 Cairo, Egypt
[8]Department of Anatomy, College of Medicine, King Khalid University, 62529 Abha, Saudi Arabia
[9]Genomics and Personalized Medicine Unit, College of Medicine, King Khalid University, 62529 Abha, Saudi Arabia
[10]Department of Basic Medical Sciences, College of Medicine, Taibah University, 42353 Madinah, Saudi Arabia
[11]Department of Andrology, Faculty of Medicine, Cairo University, 12613 Cairo, Egypt
*Correspondence: Basma.emad@kasralainy.edu.eg; doctor.basma@hotmail.com (Basma Emad Aboulhoda)

Published: 20 May 2024

Background: Infertility is a worldwide medical issue in which infection is recognized to play a major role. Pathogens trigger various mechanisms that impact fertility, either directly by affecting the physiological indices of semen or indirectly by disrupting the process of spermatogenesis. In the current work, the effect of *in-vitro* cultivation of *Escherichia coli* (*E. coli*), *Candida non-albicans* (*C. non-albicans*), and *Trichomonas vaginalis* (*T. vaginalis*) (as the most frequently reported sexually transmitted infections) was assessed on the physiological functions of the spermatozoa and the chemical characteristics of the seminal fluid.
Method: The semen samples were exposed to cultures of *E. coli*, *C. non-albicans*, and *T. vaginalis*. The study analyzed the changes in motility, agglutination, viability, DNA fragmentation index (DFI%), seminal pH, and biochemical parameters at 1/2, 1, 1.5, 2, 2.5, 3.5 and 4 hours.
Results: Incubation of the semen samples with *E. coli* resulted in a progressive increase in agglutination, pH, and nitrite. The seminal glucose and the sperm motility, on the other hand, were reduced. The sperm vitality and seminal protein remained unaffected. *C. non-albicans* induced three forms of agglutination (head-to-head, tail-to-tail, and head-to-tail), lowered pH values and decreased the sperm motility, but did not alter the seminal protein, glucose, nitrite, nor the spermatozoa viability at the different tested time intervals. *T. vaginalis* resulted in increased seminal protein, and reduced glucose, pH, and motility. It also induced minimal agglutination and caused unchanged nitrite and sperm viability. The DFI% was increased in all pathogens with the *C. non-albicans* showing the highest DNA fragmentation index.
Conclusion: Urogenital infection with *E. coli*, *C. non-albicans*, or *T. vaginalis* is assumed to affect the quality of semen through DNA fragmentation, agglutination and altered seminal chemical microenvironment.

Keywords: *E. coli*; *C. non-albicans*; *T. vaginalis*; seminal parameters; DNA fragmentation

Introduction

Infertility is a crucial global medical problem [1]. The male factor has been recognized to account for 20% of the infertility cases [2] with the genitourinary infection representing a significant cause (8–35%) [3]. Various microbial agents including bacteria, fungi, and parasites can cause reproductive disorders. These microbial infections inhibit the acrosomal functionality of spermatozoa, alter the normal sperm morphology and compromise spermatogenesis [4].

Escherichia coli (*E. coli*) is the most common bacteria affecting the urinary tract. *E. coli* is involved in causing non-sexual epididymo-orchitis, thereby representing a major causative agent of infertility [5]. This effect could be related to the toxins and metabolites produced by *E. coli*, or the direct adherence of the bacteria to the spermatozoa [6]. In Egypt, several studies were conducted alarming the epidemiological spread of *E. coli* pathogenicity [7–11].

Candida non-albicans (*C. non-albicans*) is an opportunistic fungal infection that causes urogenital infections [12]. The actual role of yeast has scarcely been investigated due to experimental difficulties [13], yet, the deleterious effects of genital *Candida* infection on semen parameters have recently attracted attention [14]. Urogenital candidiasis has significantly shifted from the *albicans* to the *non-albicans* type in Egyptian patients [15].

Trichomonas vaginalis (*T. vaginalis*) has long been recognized as a mere vaginal parasite. However, it has now been accepted as a cause of mutual urogenital infection in both males and females, representing a significant factor in sexually transmitted diseases (STDs). In men, *T. vaginalis* infection has been connected to infertility through impairment of the functionality and quality of the spermatozoa [16]. Semen was found to provide an adequate medium for the survival of trichomonads [17]. The relationship between *T. vaginalis* and infertility in females has also been reported [18].

The spermatozoon is assumed to be the only cell that accomplishes its physiological functions outside the human body. Seminal plasma is composed of sugars, and proteins in addition to other components such as $HCO_3{}^-/CO_2$, cholesterol, phospholipids, steroids, nitrogenous bases and amino acids. The optimum composition of this seminal microenvironment is crucial for optimizing sperm functions. Pathogens affect the chemical composition of semen and thus can influence the functionality of spermatozoa [19,20]. In addition, the integrity of the paternal DNA in the spermatozoa plays a significant role in initiating and preserving viable gestation, whether via natural conception or *in-vitro* fertilization [21]. Therefore, the WHO has demonstrated the importance of assessing sperm chromatin integrity and considered it an important parameter in semen analysis [22].

In this context, the current study has been designed to investigate the effect of *E. coli*, *C. non-albicans*, and *T. vaginalis* (as the most prevalent community-based transmitted organisms) on the semen functional and chemical parameters and to determine the integrity of the sperm DNA after sperm-pathogen co-incubation.

Materials and Methods

Study Setting

This study was approved by the ethical committee of the Armed Forces College of Medicine, Cairo, Egypt (No. 265 dated 17/6/2023). According to Amsterdam's declarations, all participants have been verbally informed, and a written consent has been signed before any step. All the study procedures followed the ethical standards of the National Research Committee and the 1964 Helsinki Declaration and its ensuing regulations.

The semen ejaculates were obtained from twenty four healthy adult Egyptian males as part of their fertility evaluation. The semen samples were gathered by masturbation after sexual abstinence for 4–6 days. The samples were obtained in the clinical setting (Department of Dermatology, Venereology, and Andrology, AFCM Hospital and Laboratories of Almaza, Cairo, Egypt).

Study Design

To execute the *in-vitro* cultivation of the microorganisms with semen, 24 patients (for the acquisition of 24 healthy semen ejaculates) were involved in the study. The semen samples were randomly divided into four groups as follows:

Group 1 (6 samples): Control.

Group 2 (6 samples): Semen incubated with *E. coli*.

Group 3 (6 samples): Semen incubated with *T. vaginalis*.

Group 4 (6 samples): Semen incubated with *C. non-albicans*.

The microorganisms were obtained from the microbiology and immunology department, Faculty of Medicine, Cairo University as pure isolates.

Preparation of Semen Samples

All samples were obtained in sterile plastic cups and incubated at 37 °C for 45 min to liquefy. Semen was centrifuged for 10 min at 1500 rpm at room temperature [23]. The Mycoplasma test revealed negative results. The obtained semen samples were physically assessed for color, viscosity, volume, and pH. Using light microscopy, the count of spermatozoa per mL was calculated in five small squares within the large central square in the improved Neubauer hemocytometer (Germany) [24].

Total and progressive motility was evaluated using the computer-assisted sperm analysis device, SCA® CASA system. The morphology of the spermatozoa was evaluated using an Olympus light microscope (Olympus BX43F, Olympus Corporation, Tokyo, Japan).

The *in-vitro* cultivation process included only the healthy semen ejaculates that were microscopically devoid of trichomonads and microbiologically negative for both bacteria and fungi. The standards for healthy semen samples adopted in the current studies were illustrated in Table 1 as per the WHO criteria [22,25]. None of the participants was under antibiotic therapy at the time of the sample collection or had a history of anti-parasitic/antibacterial medication for the past 3 months. Patients with abnormal semen parameters, contaminated specimens or patients suffering from diabetes mellitus were excluded from the study [26].

In-Vitro Co-Incubation of Semen with Microorganisms

One hundred microliters from each of the *E. coli*, *C. non-albicans*, and *T. vaginalis* cultures (grown for 24 and 48 hours) were mixed with an equal volume of semen samples and incubated at 37 °C in Eppendorf tubes labeled for

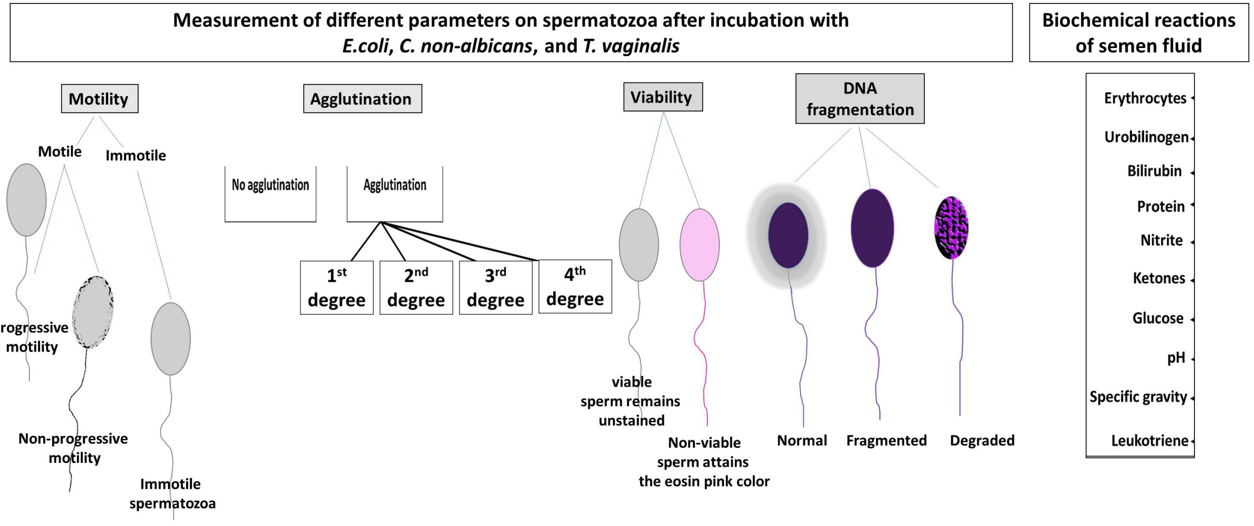

Fig. 1. The paradigm of the different measured parameters was influenced by *Escherichia coli* (*E. coli*), *Candida non-albicans* (*C. non-albicans*), and *Trichomonas vaginalis* (*T. vaginalis*). The figure was generated by the PowerPoint presentation program (version 16, Microsoft, Los Angeles, CA, USA).

Table 1. Standards for healthy semen samples with a reference range of spermatozoa motility, vitality, and count according to the WHO 2021 (the 6th edition).

Semen parameter in (%)	Reference range
Total motility	42 (40–43)
Progressive motility	30 (29–31)
Non-progressive motility	1 (1–1)
Vitality	54 (50–56)
Total sperm count in ejaculate	39–928 million
Ejaculate volume	1.5–7.6 mL
Sperm concentration	15–259 million per mL

the different time intervals. The influence of genitourinary pathogenic organisms on different parameters of the semen was assessed as shown in (Fig. 1).

Preparation of E. coli

The *E. coli* isolates were grown on sterile plates of blood agar, Chocolate, and MacConkey agar using a sterilized wire loop. All plates were, then, aerobically incubated at 37 °C for 24 hours. Evidence of selective *E. coli* growth was identified by the conventional biochemical reactions. To initiate the experiment, *E. coli* suspension was adjusted approximately at 3×10^6 bacteria/mL in sterile saline (0.85%). Cultures (Brain heart infusion broth, BHI) were finally obtained after 24 and 48 hours.

Preparation of C. non-albicans

C. non-albicans isolates were grown on Sabouraud's dextrose agar (Code: PO0394, Oxoid, Milan, Italy) at 37 °C, overnight, in aerobic conditions, and identified by the API Candida system (version 2.1, bioMerieux SA, Marcy-l'Etoile, France). The *C. non-albicans* suspension was freshly prepared the day before the experiment by incubation at 37 °C, overnight, in brain heart infusion under aerobic conditions. A final suspension, containing 4×10^6 CFU/mL, was prepared in physiological saline, starting from an initial concentration of 1×10^8 CFU/mL obtained using a 0.5 McFarland standard. The exact concentration of the suspension was verified by a spectrophotometer [13].

Preparation of T. vaginalis

The trichomonads were cultured into fresh Diamond's medium supplemented with antimicrobials (5 Ig/mL of fungizone, 10^3 U of penicillin-G/mL, and 10^2 Ig/mL of streptomycin) until it was free of bacteria. The trichomonads were then cultured on chocolate and mycoplasma agar (Oxoid) for 48 hours in 5% CO_2 at 37 °C. For the experiment, the obtained axenic trichomonads were adjusted at a concentration of approximately 5×10^5 using a Neubauer hemocytometer. Only protozoa cultures with preserved jerky movement >90% ± 10 were involved and incubated in 5% CO_2 at 37 °C [27].

Assessment of the Influence of Genitourinary Pathogenic Organisms on the Functionality and Physical Parameters of the Semen

Motility

To evaluate the influence of the tested pathogens on sperm motility at diverse time intervals, twenty µL of the semen mixture was obtained on a clean microscopic slide covered with a coverslip and examined underneath an Olympus light microscope (Olympus BX43F, Olympus Corporation, Tokyo, Japan) at magnification 400×. The interpretation was defined as total motility (progressive and non-progressive) or non-motile [28].

Agglutination

The impact of different pathogens on the agglutination of spermatozoa was evaluated using an Olympus light microscope (Olympus BX43F, Olympus Corporation, Tokyo, Japan). Agglutination of motile sperms was identified as head-to-head, tail-to-tail, or head-to-tail agglutination.

Agglutination of mobile spermatozoa to each other was thoroughly examined to exclude non-specific adherence of motile spermatozoa to epithelial debris and adherence of immobile spermatozoa. The interpretation was done as follows; grade 1 (isolated): <10 spermatozoa per agglutinate with many free motile spermatozoa, grade 2 (moderate): 10–50 spermatozoa per agglutinate with many free motile spermatozoa, grade 3 (massive): agglutinates with >50 spermatozoa with only a few free motile spermatozoa, and grade 4 (complete): completely agglutinated spermatozoa with no motile free spermatozoa [25].

Viability

To evaluate the influence of the tested microorganisms on the viability of the spermatozoa, a dye exclusion test using Eosin Y 0.5% aqueous solution was implemented as an indicator dye. For this, equivalent volumes (100 µL) of each semen sample and different microorganisms were thoroughly mixed and incubated at 37 °C. A wet preparation, consisting of 10 µL of the well-mixed reaction mixture and Eosin Y (0.5%), was performed and observed under light microscopy. As a control, the semen sample was mixed and incubated with BHI broth. The results were interpreted as the percentage of live (unstained) spermatozoa and the dead (pink stained).

Biochemical Investigation of the Seminal Fluid

The impact of incubation of urogenital pathogens on the vital biochemical parameters (Protein, Nitrite, Glucose, pH) was qualitatively and semi-quantitatively assessed. The commercially available reagent strip test (Medi-Test Combi 10 ® SGL, item number: MN93067, Macherey-Nagel GmbH & Co., Düren, Germany) was completely immersed in the test tube for 2–3 seconds and immediately read. The diagnostic parameters to each index test were valued using the corresponding cut-off levels (glucose positive at >50 mg/dL, protein at >30 mg/dL whereas nitrite was negative or positive, and pH >7 is alkaline and <7 is acidic [29]. The count of the nitrite positive and nitrite negative samples was estimated using the reagent strip test(Medi-Test Combi 10 ® SGL, item number: MN93067, Macherey-Nagel GmbH & Co., Düren, Germany) after incubation of the semen samples with each of the organisms at the different tested time intervals. Quantitative analysis was done for glucose (RayBio® Glucose Colorimetric Assay Kit, Catalog #: MA-GLU, RayBiotech Inc., GA, USA), protein (colorimetric, Spectrum-Diagnostics Reagents, Catalog #: 310 001, Egyptian Company for Biotechnology, Cairo, Egypt), and pH (Lit-Control pH Meter, model number: CN175533.9, Devicare, Barcelona, Spain).

Sperm DNA Fragmentation Test

DNA fragmentation was evaluated using a Halosperm® (HT-HS10 batch number 1623, Halotech DNA SL, Madrid, Spain) as per the manufacturer's instructions. The initial acid treatment of the sperm cells denatures the fragmented molecules of the DNA. Thereafter, the lysis solution eliminates the nuclear proteins. In case there is no massive DNA damage nucleoids with large halos emergent from a central core are produced. Spermatozoa with fragmented DNA either do not express a dispersion halo or show a minimal halo from their nucleoids.

$$\text{DNA fragmentation index (DFI\%)} = \frac{\text{small halo+without halo+degraded sperm head}}{\text{total count of spermatozoa}} \times 100\%$$

Interpretation: the reference range is <25% excellent to good DNA integrity, 25–50% fair to poor, and >50% very poor DNA integrity.

Statistics

Statistical package for the Social Sciences (SPSS) version 26 (IBM Corp., Armonk, NY, USA) was used to analyze the data. Data were summarized using mean and standard deviation for quantitative variables and frequencies (number of cases) and relative frequencies (percentages) for categorical variables. Comparisons between groups were done using analysis of variance (ANOVA) with multiple comparisons post hoc Bonferroni test. For comparing categorical data, the Chi-square test was performed [30,31]. *p*-values less than 0.05 were considered statistically significant.

Results

Agglutination

Light microscopy was carried out to assess agglutination in spermatozoa after being incubated with various urogenital pathogens. The results of the control semen sample revealed normal freely mobile spermatozoa with an oval-shaped head and normal neck and tail (Fig. 2A). Incubation of spermatozoa with *E. coli* showed adherence of the bacilli to the tails and heads of the spermatozoa (Fig. 2B) resulting in progressive agglutination at different time intervals. *C. non-albicans* resulted in tail-to-tail agglutination of the spermatozoa during the first hour. Progressive adherence of *C. non-albicans* to the heads of the spermatozoa was also observed starting from the 2nd hour of incubation (Fig. 2C) with triggering of the three forms of agglutination (head-to-head, tail-to-tail, or head-to-tail). *T. vaginalis*, head-to-head agglutination was scarcely seen (Fig. 2D).

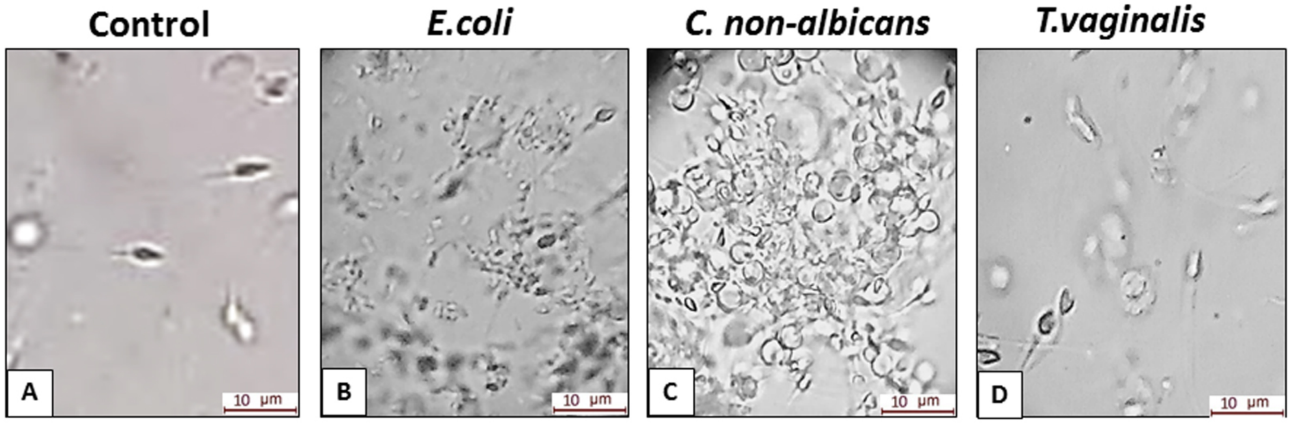

Fig. 2. Photograph showing agglutination and viability of spermatozoa in semen fluid after co-incubation with 24-hour cultures for 30 minutes. (A) Control samples, (B) *E. coli*, (C) *C. non-albicans* and (D) *T. vaginalis*. Note control sample shows no agglutination and viable sperms while the pathogen incubated samples show agglutination of spermatozoa and dead sperms (scale bar: 10 µm) (Images are captured via an Olympus light microscope (Olympus BX43F, Olympus Corporation, Tokyo, Japan).

E. coli

E. coli-48-hour culture showed significant reductions in seminal glucose (mg/dL) at ½, 3.5, and 4 hours of co-incubation compared with healthy semen (11.5 ± 2.26 versus 14.83 ± 1.47, 4 ± 1.26 versus 6.83 ± 2.48, and 2.83 ± 1.47 versus 5.83 ± 2.48 respectively) (p-value < 0.05). Seminal protein (mg/dL) was not altered at different time intervals with the *E. coli* cultures (p-value > 0.05); yet, seminal nitrite was predominantly positive (p-value < 0.05). After 4 hours of co-incubation, *E. coli* cultures resulted in increased pH values (8.8 ± 0.21 and 8.87 ± 0.16 respectively) compared with healthy semen (8.18 ± 0.51) (p-value < 0.05).

Regarding the effect of *E. coli* on agglutination at different time intervals, the first thirty minutes of co-incubation with the *E. coli* cultures showed grade 2 agglutination of spermatozoa (49.17 ± 11.14 and 55.83 ± 9.7 respectively versus 1.83 ± 1.94 in the control samples) (p-value < 0.05). By the end of the fourth hour, agglutination progressed into grade 4 (97 ± 2.61 and 98.5 ± 1.05 respectively versus 15.5 ± 2.07 in the control samples) (p-value $<$ 0.05). Compared with the healthy uninfected semen, incubation with the *E. coli* cultures exhibited a significant reduction in motility ($p < 0.05$) at different time intervals whereas, the percentage of viability was not affected ($p >$ 0.05) (Fig. 3).

C. non-albicans

The *C. non-albicans* cultures did not result in significant alterations in the seminal protein and glucose at all the tested time intervals. At 3 and 4 hours of co-incubation, the *C. non-albicans*-24-hour culture showed lower pH values (7.7 ± 0.11, 7.57 ± 0.1, and 7.4 ± 0.13 respectively) versus healthy semen (8.18 ± 0.51) with p-value < 0.05. Also, the *C. non-albicans*-48-hour culture exhibited reduced pH values at 3.5 hours and 4 hours of co-incubation (7.62 ± 0.08 and 7.45 ± 0.1, respectively) compared with the healthy semen ($p < 0.05$).

Incubation of the semen samples with *C. non-albicans* revealed a significant increase in agglutination grade to grade 3 (84.17 ± 8.61) and grade 4 (95.5 ± 3.27) agglutination after 4 hours of co-incubation with the 24-hour and 48-hour cultures respectively ($p < 0.05$). The *C. non-albicans* 24-hour and 48-hour cultures resulted in a significant reduction in spermatozoal motility ($p < 0.05$) at the different tested time intervals while the percentage of sperm viability was not significantly altered ($p > 0.05$) (Fig. 4).

T. vaginalis

Incubation of the semen with the 24-hour and 48-hour *T. vaginalis* cultures resulted in significant reductions in seminal glucose (mg/dL) within the 4-hour incubation period (p-value <0.05). Also, the *T. vaginalis*-48-hour culture showed a significant increase in seminal protein (mg/dL) after 1.5 hours of co-incubation.

The pH values were also significantly reduced with both the 24-hour and 48-hour *T. vaginalis* cultures at 2 to 4 hours of semen co-incubation. Through the experiment, the seminal nitrite was not altered with neither the 24-hour nor the 48-hour *T. vaginalis* cultures.

Sperm agglutination was scarcely seen with both the 24-hour and 48-hour cultures of *T. vaginalis* and was almost always isolated agglutination (grade 1) with a statistically insignificant mean difference relative to the control samples. The 24-hour and 48-hour *T. vaginalis* cultures also resulted in a significant reduction in sperm motility relative to the healthy uninfected semen ($p < 0.05$) at the different tested time intervals. Nevertheless, viability was not affected (Fig. 5).

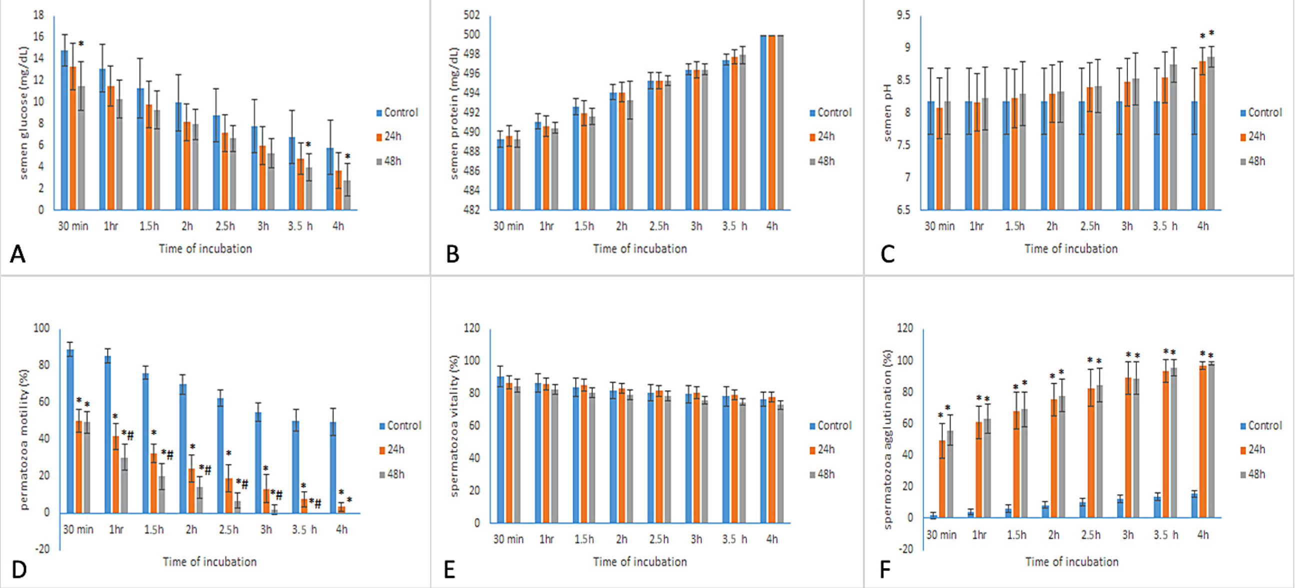

Fig. 3. Alterations in semen parameters following *in-vitro* cultivation with the *E. coli* cultures at different time intervals. (A) Semen glucose. (B) Semen protein. (C) Semen pH. (D) Spermatozoa motility. (E) Spermatozoa vitality. (F) Spermatozoa agglutination. Data are represented as mean ± SD. (*) Statistically significant compared to the healthy control (n = 6). (#) Statistically significant compared with 24 hours at $p < 0.05$ using analysis of variance (ANOVA) multiple comparisons with post hoc Bonferroni test.

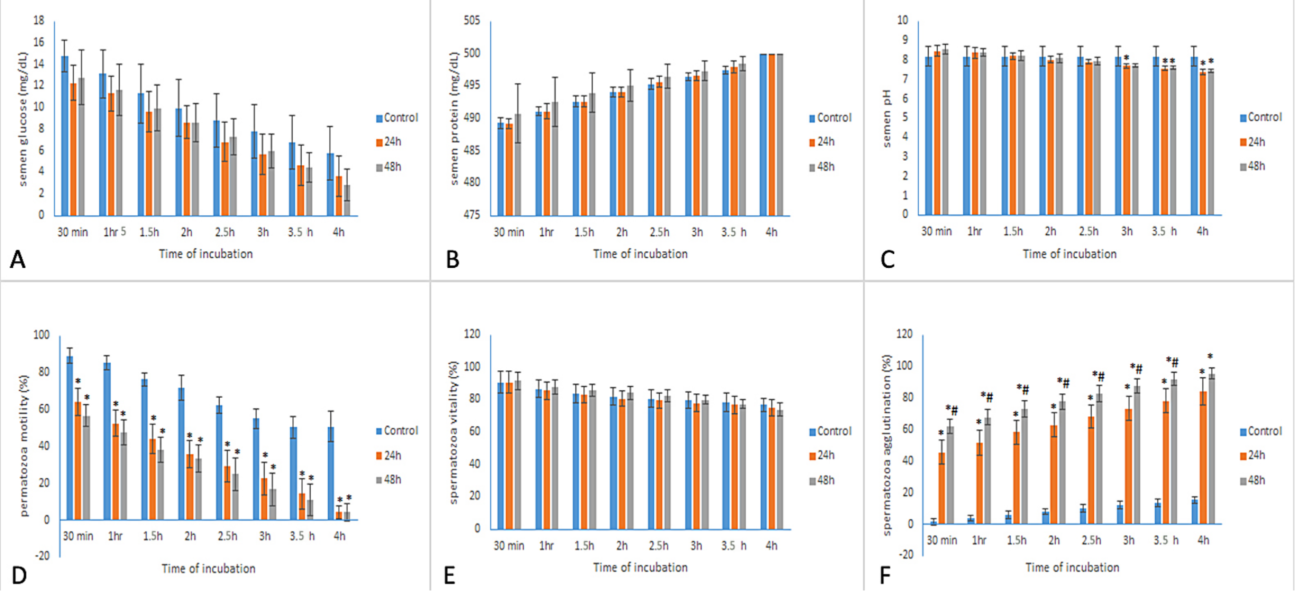

Fig. 4. Alterations in semen parameters following *in-vitro* cultivation with *C. non-albicans* cultures at different time intervals. (A) Semen glucose. (B) Semen protein. (C) Semen pH. (D) Spermatozoa motility. (E) Spermatozoa vitality. (F) Spermatozoa agglutination. Data are represented as mean ± SD. (*) Statistically significant compared to the healthy control (n = 6). (#) Statistically significant compared with 24 hours at $p < 0.05$ using ANOVA multiple comparisons with post hoc Bonferroni test.

DNA Fragmentation

The spermatozoa incubated with the urogenital pathogens in the current study showed high DFI% indicating poor integrity of the DNA molecule. *C. non-albicans* co-incubated with semen (group 2) showed the highest DFI% (97%) (p-value < 0.05) with thirty percent having degraded DNA whereas a big halo in non-fragmented DNA was as low as 1%. Nevertheless, there were no significant differences in the average DFI% between sperms incubated with *E. coli* (group 3) (56%) or with trichomonads (57%) (group 4) (p-value > 0.05) (Fig. 6). Values of fragmented DNA (small halo, without halo, and degraded) were also evaluated.

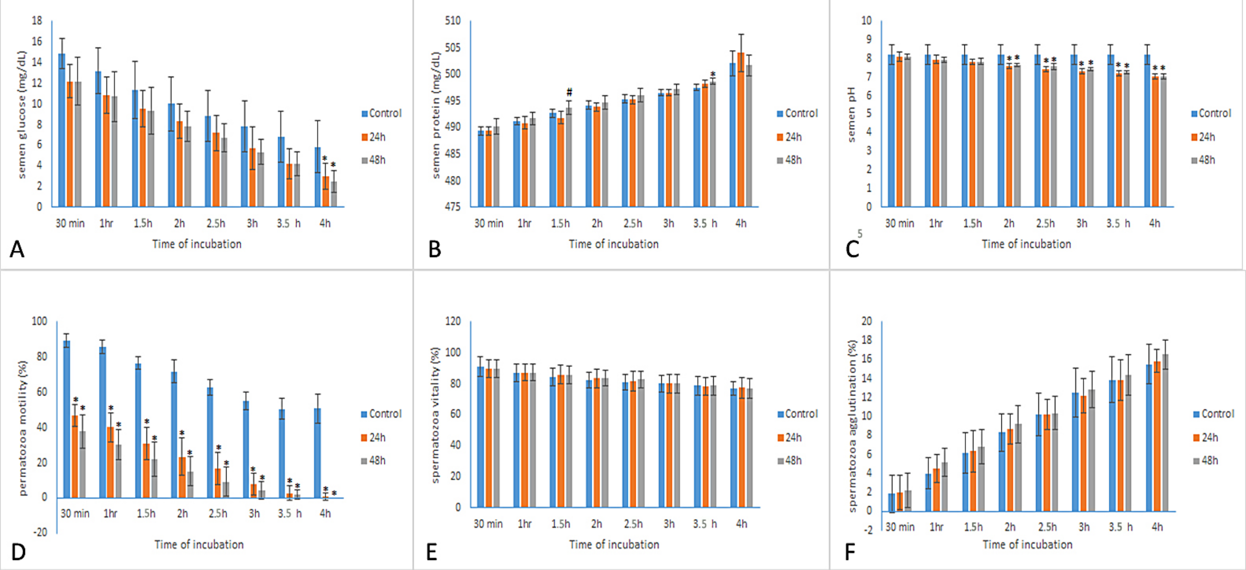

Fig. 5. Alterations in semen parameters following *in-vitro* cultivation with 24-hour and 48-hour-old cultures of *T. vaginalis* at different time intervals. (A) Semen glucose. (B) Semen protein. (C) Semen pH. (D) Spermatozoa motility. (E) Spermatozoa vitality. (F) Spermatozoa agglutination. Data are represented as mean ± SD (n = 6). (*) Statistically significant compared to the healthy control, (#) Statistically significant compared with 24 hours at $p < 0.05$ using ANOVA multiple comparisons with post hoc Bonferroni test.

Discussion

The current study has been designed to investigate the effect of *in-vitro* incubation of pathogens on the semen functional and chemical parameters. *E. coli* induced progressive agglutination and clustering of spermatozoa in a time-dependent manner. *E. coli* is the most frequent bacteria to induce agglutination of spermatozoa (54.7%) followed by *Proteus* sp., *Streptococcus agalactiae*, and *Enterococcus fecalis*. This reflects the great capability of *E. coli* to directly interact with the sperms [32,33].

E. coli co-incubation with semen in the current study also resulted in increased pH values. This alkaline pH suppresses the cell division protein (FtsN) in *E. coli* because *E. coli* requires a Na^+/H^+ antiporter to proliferate at alkaline pH [34].

Nitrite increased predominantly in the semen incubated with *E. coli*. This could be explained by the ability of *E. coli* to import Nitrate and reduce it into nitrite. *E. coli* then expels nitrite and reduces it into ammonia. This mechanism protects the cytoplasm of *E. coli* from extreme nitrite toxicity [35]. Increased nitrite in seminal fluid has been previously linked to oligo- and azoospermic [36].

Seminal glucose was also reduced following the co-incubation of semen with *E. coli* in the current study. This is concordant with the previous reports illustrating the strong association between glucose consumption and energy production with the oxidation-reduction reactions, and growth of *E. coli* [37].

In the current work, the 48-hour culture of *E. coli* resulted in a significant reduction in spermatozoa motility when compared with the 24-hour culture and control. This could be attributed to the soluble factors produced by *E. coli* which can affect sperm functions [38]. The effect of *E. coli* on spermatozoa is dependent on the bacterial load [39,40].

The current work revealed no significant differences in the protein concentration nor the viability of spermatozoa between the *E. coli*-incubated and the control semen samples. The Semenogelin (Sg) protein, a major constituent of the semen coagulum, activates the hyaluronidase enzyme inducing an anti-microbial effect. “Eppin” is another seminal protein component that induces antibacterial activity through alteration of the permeability of the inner and outer membranes of *E. coli*, thereby protecting spermatozoa [41,42].

DNA fragmentation increased up to 56% after incubation with *E. coli* (p-value < 0.05). This could be attributed to the capacity of *E. coli* to directly attach to the sperms and their ability to trigger oxidative stress and apoptotic damage [4]. In a prior study, comparable deterioration in semen quality was recorded after incubation with different concentrations of enterotoxigenic and verotoxigenic *E. coli* [43].

Candida non-albicans cultures in the current study resulted in a significant reduction in the motility of spermatozoa at the different tested time intervals. *Candida sp.* has been previously proven to directly adhere to the sperms and damage their ultrastructure [44]. *Candida* also releases extracellular sperm immobilization factors that hinder sperm motility [45]. Co-incubation with *C. non-albicans* in the current study also induced marked sperm agglutination (tail-to-tail, head-to-head, and head-to-tail). Agglutination is always associated with morphological changes in sper-

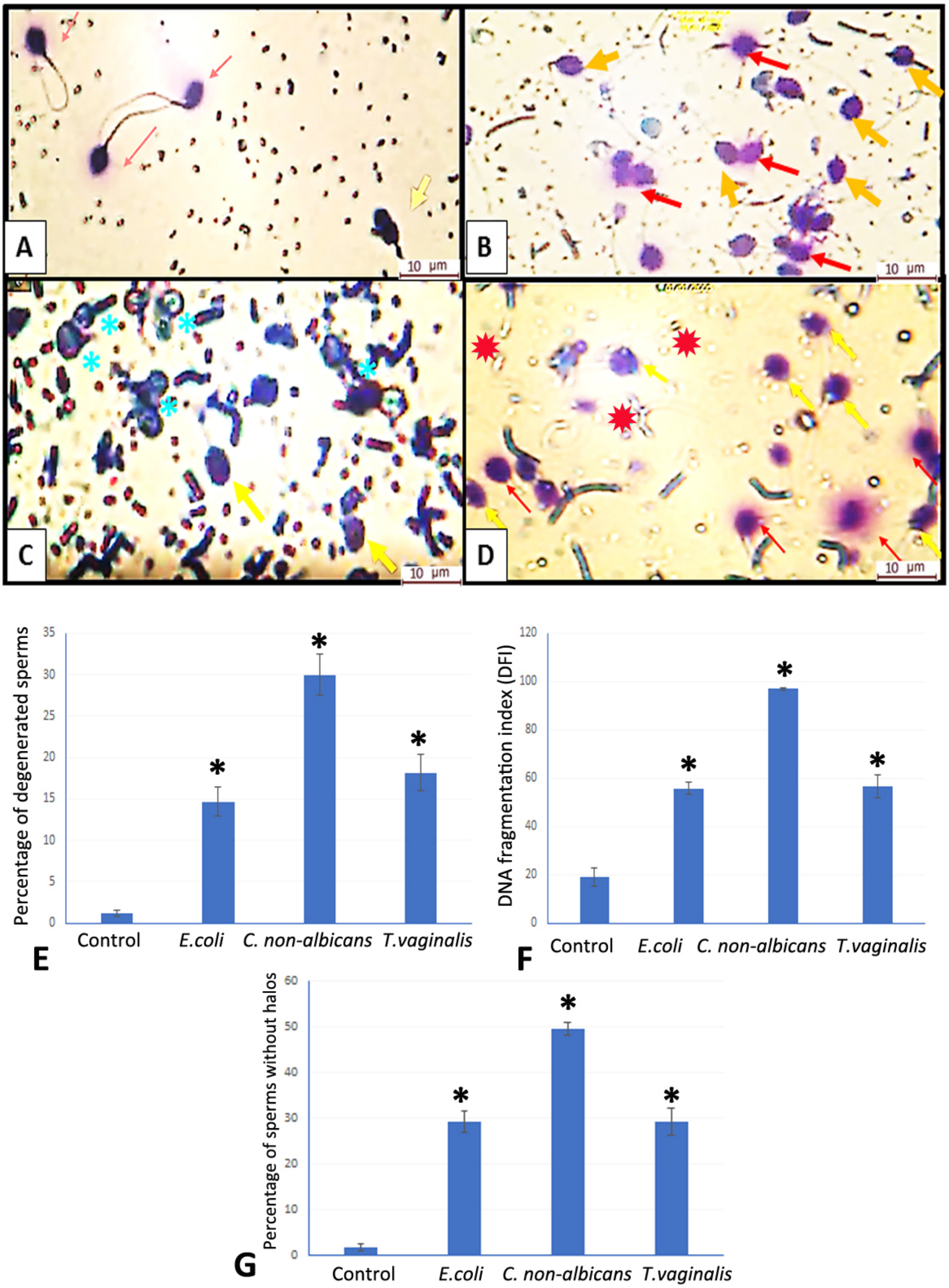

Fig. 6. DNA fragmentation after 4 hours of co-incubation with the 24-hour cultures. (A) Control. (B) *E. coli*. (C) *C. non-albicans*. (D) *T. vaginalis*. Note: Blue asterisks point to *C. non-albicans*, red asterisks point to degraded sperms with pin and double-head, yellow arrows point to sperms without halo, and red arrows point to sperm with a big or medium halo (scale bar: 10 μm). (E) Percentage of degenerated sperms. (F) DNA fragmentation index (DFI%). (G) Percentage of sperms without halos. Data are represented as mean ± SD (n = 6). (*) Statistically significant compared to the healthy control at $p < 0.05$ using ANOVA multiple comparisons with post hoc Bonferroni test. Images are captured via an Olympus light microscope (Olympus BX43F, Olympus Corporation, Tokyo, Japan).

matozoa, with essential interfering with sperm motility. This agglutination effect is also dependent on the incubation time and the concentration of the organisms [13,46].

In the current work, the pH of semen has substantially dropped following co-incubation with *C. non-albicans* in a time-dependent manner. The alkaline seminal pH preserves the quality and viability of the spermatozoa [47]. Any modification of the microenvironment of seminal plasma leads to excessive energy consumption and deterioration of sperm motility [48].

In the current study, co-incubation with *C. non-albicans* increased the DFI% up to 97%. DNA fragmentation is closely associated with sperm apoptosis [49] and is regulated by a caspase-regulated DNase complex known as the DNA fragmentation factor [50]. DNA fragmentation predisposes to oxidative stress [51] and latent chromosomal damage [52] because it is closely related to condensation of chromatin material, nuclear fragmentation, and sperm apoptosis [53]. Detection of *Candida* infection has been proposed as an essential factor to avoid poor pregnancy outcomes [54,55].

In the current study, agglutination was hardly seen with the *T. vaginalis* cultures. Despite the ability of *T. vaginalis* to induce tropism of the spermatozoa (for the purpose of phagocytosis), yet, the trichomonads undergo immediate necrosis when they come in contact with the sperms [56]. This comes in agreement with our results which revealed that prokaryotic organisms are more efficient in triggering agglutination rather than the eukaryotic *Trichomonas*.

In the current study, motility was markedly reduced upon incubation with *T.* vaginalis. *T. vaginalis* produces extracellular polymeric substances (EPS) that exert an inhibitory effect on semen motility [57]. Also, the rotation of *T. vaginalis* trophozoites hinders the horizontal movement of the spermatozoa [58]. Co-incubation of semen with *T. vaginalis* cultures showed a reduction in seminal PH. Low pH in semen impairs the cell membrane of the sperm or increases the active content of oxygen, thus in turn distresses the motility of sperm and its capacitation [59].

T. vaginalis co-incubation in the current study resulted in a reduction of the seminal glucose. *T. vaginalis* is recognized to gather considerable amounts of the polysaccharide glycogen that serves as a carbon and an energy stock. Yet, glycogen stores are dependent on the extracellular concentrations of glucose [60]. Glucose is the chief source of ATP generation in the hydrogenosome of the parasite [61]. Interestingly, survival of *T. vaginalis* was found to be enhanced in a glucose-restricted microenvironment where it could benefit from low glucose levels [62]. A recent study demonstrated the presence of Na^+/K^+-ATPase in the sperm flagellar membrane. This Na^+/K^+-ATPase is important for the uptake of glucose, production of ATP, and induction of sperm motility [63]. This could explain the relation between the reduction of seminal glucose and the impaired sperm motility observed with the *T. vaginalis* cultures in the current study.

Another remarkable observation in the current study was the increased seminal protein after co-incubation with *T. vaginalis*. A previous study has reported that *T. vaginalis* produces protein metabolites that increase the viscosity of seminal fluid and exert a lethal effect on the sperms [64].

The semen samples incubated with *T. vaginalis* in our study recorded a 57% increase in the DFI%. This could be attributed to the excretory/secretory metabolites produced by *T. vaginalis* that induce DNA fragmentation, reduce mitochondrial membrane potential, and initiate sperm apoptosis [65]. Our work revealed that co-incubation with *T. vaginalis*, on the other hand, did not exhibit alteration in spermatozoa viability. The extracellular polymeric substances produced by *T. vaginalis* have been previously reported to affect the viability of sperm in a time-dependent manner [57].

Limitations of the current study are to be considered. We recommend a wider scale of studies on the genome of *C. non-albicans*, *T. vaginalis*, and *E coli*. Care should also be attained during the interpretation of the *in-vitro* studies into the clinical practice since the situation of genitourinary infections could differ significantly from the *in-vitro* incubation of microorganisms. Future studies should be designed to analyze the semen chemical and functional parameters among different genitourinary infection patients.

Conclusion

Incubation of semen with *E. coli* resulted in a significant reduction in seminal glucose and an increase in seminal pH. The seminal protein remained unaltered throughout the experiment; yet, the seminal nitrite was positive. *E. coli* also induced agglutination through its attachment to the tail of spermatozoa resulting in progressive agglutination that proceeded into grade 4 by the end of the fourth hour. Sperm motility was significantly reduced while viability was not affected. *C. non-albicans* cultures did not show alterations in seminal protein, glucose, nor viability of the spermatozoa. Yet, pH and motility were reduced compared to the control semen samples. Also, *C. non-albicans* resulted in grade 4 agglutination with its three forms (head-to-head, tail-to-tail, or head-to-tail agglutination). *T. vaginalis* reduced seminal glucose, pH, and motility and increased seminal protein. Nitrite and viability appeared not to be affected, and agglutination was scarcely seen. The DFI% was increased in all the tested organisms, yet, the *C. non-albicans* exhibited the highest DNA fragmentation index. The increase in DFI% was statistically non-significant in both *E. coli* and *T. vaginalis*.

Availability of Data and Materials

Data are available upon reasonable request from the corresponding author.

Author Contributions

MA and EAES: designed the research study. RYS, HE, ASG, MFF, BEA and AM: acquisition of data, analysis and interpretation of data. HMA and EAA: conceptualization. All authors contributed to the drafting and critical revision of the manuscript. All authors have read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by the ethical committee of the Armed Forces College of Medicine, Cairo, Egypt (No. 265 dated 17/6/2023). All participants have been informed verbally and a written consent has been signed before any step according to Amsterdam's declarations. All procedures involved in the current study were performed following the ethical standards of the National Research Committee and with the 1964 Helsinki Declaration and its ensuing regulations.

Acknowledgment

Not applicable.

Funding

The authors extend their appreciation to the Deanship of Scientific Research at King Khalid University for funding this work through a large group Research Project under grant number RGP2/302/44.

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Deshpande PS, Gupta AS. Causes and Prevalence of Factors Causing Infertility in a Public Health Facility. Journal of Human Reproductive Sciences. 2019; 12: 287–293.

[2] Rumbold AR, Sevoyan A, Oswald TK, Fernandez RC, Davies MJ, Moore VM. Impact of male factor infertility on offspring health and development. Fertility and Sterility. 2019; 111: 1047–1053.

[3] Jebur MS. The Effect of Gram Negative Bacteria on Semen Parameters of A Sample of Infertile Men and Antibiotic Susceptibility Tests. Journal of Techniques. 2022; 4.

[4] Henkel R, Offor U, Fisher D. The role of infections and leukocytes in male infertility. Andrologia. 2021; 53: e13743.

[5] Klein B, Bhushan S, Günther S, Middendorff R, Loveland KL, Hedger MP, *et al*. Differential tissue-specific damage caused by bacterial epididymo-orchitis in the mouse. Molecular Human Reproduction. 2020; 26: 215–227.

[6] Bonet S, Delgado-Bermúdez A, Yeste M, Pinart E. Study of boar sperm interaction with Escherichia coli and Clostridium perfringens in refrigerated semen. Animal Reproduction Science. 2018; 197: 134–144.

[7] Hassuna NA, Khairalla AS, Farahat EM, Hammad AM, Abdel-Fattah M. Molecular characterization of Extended-spectrum β lactamase- producing E. coli recovered from community-acquired urinary tract infections in Upper Egypt. Scientific Reports. 2020; 10: 2772.

[8] Abd El-Baky RM, Ibrahim RA, Mohamed DS, Ahmed EF, Hashem ZS. Prevalence of Virulence Genes and Their Association with Antimicrobial Resistance Among Pathogenic *E. coli* Isolated from Egyptian Patients with Different Clinical Infections. Infection and Drug Resistance. 2020; 13: 1221–1236.

[9] Esmaeel NE, Gerges MA, Hosny TA, Ali AR, Gebriel MG. Detection of Chromosomal and Plasmid-Mediated Quinolone Resistance Among *Escherichia coli* Isolated from Urinary Tract Infection Cases; Zagazig University Hospitals, Egypt. Infection and Drug Resistance. 2020; 13: 413–421.

[10] Ali GH, Yakout MA. Comparative Study of ESBL Production Among Uropathogenic Escherichia coli Clinical Isolates from Pre- and Post-menopausal Women in Egypt. Current Microbiology. 2021; 78: 3516–3525.

[11] El-Baz R, Said HS, Abdelmegeed ES, Barwa R. Characterization of virulence determinants and phylogenetic background of multiple and extensively drug resistant Escherichia coli isolated from different clinical sources in Egypt. Applied Microbiology and Biotechnology. 2022; 106: 1279–1298.

[12] Rolo J, Caixeirinho P, Gomes-Ruivo P, Gaspar C, Fernandes I, Palmeira-de-Oliveira R, *et al*. Semen supports growth of Candida albicans: A putative risk factor for recurrence of vulvovaginal infections? The Journal of Obstetrics and Gynaecology Research. 2020; 46: 1893–1899.

[13] Castrillón-Duque EX, Puerta Suárez J, Cardona Maya WD. Yeast and Fertility: Effects of *In Vitro* Activity of *Candida* spp. on Sperm Quality. Journal of Reproduction & Infertility. 2018; 19: 49–55.

[14] Khalaf Y, Farrag A, Shaeer EKZ. Effect of Female Genital Candidiasis on Semen Parameters Female Genital Candidiasis and Semen. Kasr Al Ainy Medical Journal. 2022; 28: 32–37.

[15] Dahroug HM, Salem DA, Tarek B, Gamal D, Helmy H, El-Defrawy I, *et al*. Speciation and Antifungal Susceptibility of Clinical Non-albicans Candida isolated from Patients in a Tertiary Care Centre in Egypt. Egyptian Journal of Medical Microbiology. 2023; 32: 23–31.

[16] Mielczarek E, Blaszkowska J. Trichomonas vaginalis: pathogenicity and potential role in human reproductive failure. Infection. 2016; 44: 447–458.

[17] Barbosa MDS, Andrade de Souza IB, Schnaufer ECDS, Silva LFD, Maymone Gonçalves CC, Simionatto S, *et al*. Prevalence and factors associated with Trichomonas vaginalis infection in indigenous Brazilian women. PLOS ONE. 2020; 15: e0240323.

[18] Hashemi N, Soleimani Z. The Relation Between Trichomonas Vaginalis and Female Infertility: A Meta-Analysis. Journal of Obstetrics, Gynecology and Cancer Research. 2022; 7: 513–517.

[19] Hussein AH, Saleh MH, Nagaty IM, A Ghieth K, El-Azab NA. Prevalence, Clinical Criteria and Sociodemographic Predictors of Trichomonas vaginalis Infection in Suspected Egyptian Women, Using Direct Diagnostic Techniques. Iranian Journal of Parasitology. 2015; 10: 432–440.

[20] MacDonald AA, Herbison GP, Showell M, Farquhar CM. The impact of body mass index on semen parameters and reproductive hormones in human males: a systematic review with meta-analysis. Human Reproduction Update. 2010; 16: 293–311.

[21] Fernández JL, Muriel L, Goyanes V, Segrelles E, Gosálvez J, Enciso M, *et al*. Halosperm is an easy, available, and cost-effective alternative for determining sperm DNA fragmentation. Fertility and Sterility. 2005; 84: 860.

[22] Boitrelle F, Shah R, Saleh R, Henkel R, Kandil H, Chung E, *et al*. The Sixth Edition of the WHO Manual for Human Semen

Analysis: A Critical Review and SWOT Analysis. Life. 2021; 11: 1368.

[23] Björndahl L, Kirkman Brown J, other Editorial Board Members of the WHO Laboratory Manual for the Examination and Processing of Human Semen. The sixth edition of the WHO Laboratory Manual for the Examination and Processing of Human Semen: ensuring quality and standardization in basic examination of human ejaculates. Fertility and Sterility. 2022; 117: 246–251.

[24] Rehman SU, Shafique L, Yousuf MR, Liu Q, Ahmed JZ, Riaz H. Spectrophotometric calibration and comparison of different semen evaluation methods in Nili-Ravi buffalo bulls. Pakistan Veterinary Journal. 2019; 39: 2074–7764.

[25] Franken DR, Oehninger S. Semen analysis and sperm function testing. Asian Journal of Andrology. 2012; 14: 6–13.

[26] Temidayo SO, Du Plessis SS. Diabetes mellitus and male infertility. Asian Pacific Journal of Reproduction. 2018; 7: 6–14.

[27] Borchardt KA, Zhang MZ, Shing H, Flink K. A comparison of the sensitivity of the InPouch TV, Diamond's and Trichosel media for detection of Trichomonas vaginalis. Genitourinary Medicine. 1997; 73: 297–298.

[28] Cooper TG, Noonan E, von Eckardstein S, Auger J, Baker HWG, Behre HM, *et al*. World Health Organization reference values for human semen characteristics. Human Reproduction Update. 2010; 16: 231–245.

[29] El Saftawy EA, Abd El-Aal AA, Badawi M, Attia SS, Abdelraouf A, Shoeib EY, *et al*. Research Note. One minute, intraoperative assessment of the viability of hydatid cysts using a simple reagent strip test. Helminthologia. 2017; 54: 157–164.

[30] Chan YH. Biostatistics 102: quantitative data–parametric & non-parametric tests. Singapore Medical Journal. 2003; 44: 391–396.

[31] Chan YH. Biostatistics 103: qualitative data - tests of independence. Singapore Medical Journal. 2003; 44: 498–503.

[32] Zeyad A, Amor H, Hammadeh ME. The impact of bacterial infections on human spermatozoa. International Journal of Women's Health and Reproduction Sciences. 2017; 5: 243–252.

[33] Padmaja N, Pilli R. Role of Microbial Agents in Reproductive Health. JK Science: Journal of Medical Education & Research. 2021; 23: 106–110.

[34] Mueller EA, Westfall CS, Levin PA. pH-dependent activation of cytokinesis modulates Escherichia coli cell size. PLOS Genetics. 2020; 16: e1008685.

[35] Wang C, Chao Y, Matera G, Gao Q, Vogel J. The conserved 3' UTR-derived small RNA NarS mediates mRNA crossregulation during nitrate respiration. Nucleic Acids Research. 2020; 48: 2126–2143.

[36] Battaglia C, Giulini S, Regnani G, Di Girolamo R, Paganelli S, Facchinetti F, *et al*. Seminal plasma nitrite/nitrate and intratesticular Doppler flow in fertile and infertile subjects. Human Reproduction. 2000; 15: 2554–2558.

[37] Millard P, Smallbone K, Mendes P. Metabolic regulation is sufficient for global and robust coordination of glucose uptake, catabolism, energy production and growth in Escherichia coli. PLoS Computational Biology. 2017; 13: e1005396.

[38] Schulz M, Sánchez R, Soto L, Risopatrón J, Villegas J. Effect of Escherichia coli and its soluble factors on mitochondrial membrane potential, phosphatidylserine translocation, viability, and motility of human spermatozoa. Fertility and Sterility. 2010; 94: 619–623.

[39] So KM, Sa SJ, Kim HJ, Chung KH, Jung BY, Son JH, *et al*. Effects of Escherichia coli contamination on extended porcine semen parameters. Reproductive and Developmental Biology. 2011; 35: 479–483.

[40] Pinart E, Domènech E, Bussalleu E, Yeste M, Bonet S. A comparative study of the effects of Escherichia coli and Clostridium perfringens upon boar semen preserved in liquid storage. Animal Reproduction Science. 2017; 177: 65–78.

[41] de Lamirande E. Semenogelin, the main protein of the human semen coagulum, regulates sperm function. Seminars in Thrombosis and Hemostasis. 2007; 33: 60–68.

[42] Yenugu S, Richardson RT, Sivashanmugam P, Wang Z, O'rand MG, French FS, *et al*. Antimicrobial activity of human EPPIN, an androgen-regulated, sperm-bound protein with a whey acidic protein motif. Biology of Reproduction. 2004; 71: 1484–1490.

[43] Bussalleu E, Yeste M, Sepúlveda L, Torner E, Pinart E, Bonet S. Effects of different concentrations of enterotoxigenic and verotoxigenic E. coli on boar sperm quality. Animal Reproduction Science. 2011; 127: 176–182.

[44] Tian YH, Liu J, Xu HM, Hu L, Xiong CL. Influence of Candida albicans on the motility and ultrastructure of human spermatozoa *in vitro*. National Journal of Andrology. 2005; 11: 179–184.

[45] Kumari S, Chauhan A, Thaper D, Prabha V. Sperm immobilization factor of Candida albicans: A proposed mechanism of infertility in female mice. Global Journal of Fertility and Research. 2020; 5: 001–006.

[46] Kaur K, Prabha V. Spermagglutinating Escherichia coli and its role in infertility: *in vivo* study. Microbial Pathogenesis. 2014; 69–70: 33–38.

[47] Zhou J, Chen L, Li J, Li H, Hong Z, Xie M, *et al*. The Semen pH Affects Sperm Motility and Capacitation. PLOS ONE. 2015; 10: e0132974.

[48] Berktas M, Aydin S, Yilmaz Y, Cecen K, Bozkurt H. Sperm motility changes after coincubation with various uropathogenic microorganisms: an *in vitro* experimental study. International Urology and Nephrology. 2008; 40: 383–389.

[49] Sánchez-Martín P, Dorado-Silva M, Sánchez-Martín F, González Martínez M, Johnston SD, Gosálvez J. Magnetic cell sorting of semen containing spermatozoa with high DNA fragmentation in ICSI cycles decreases miscarriage rate. Reproductive Biomedicine Online. 2017; 34: 506–512.

[50] Slee EA, Adrain C, Martin SJ. Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. The Journal of Biological Chemistry. 2001; 276: 7320–7326.

[51] Dorostghoal M, Kazeminejad SR, Shahbazian N, Pourmehdi M, Jabbari A. Oxidative stress status and sperm DNA fragmentation in fertile and infertile men. Andrologia. 2017; 49: e12762.

[52] Santiso R, Tamayo M, Gosálvez J, Johnston S, Mariño A, Fernández C, *et al*. DNA fragmentation dynamics allows the assessment of cryptic sperm damage in human: evaluation of exposure to ionizing radiation, hyperthermia, acidic pH and nitric oxide. Mutation Research. 2012; 734: 41–49.

[53] Majtnerová P, Roušar T. An overview of apoptosis assays detecting DNA fragmentation. Molecular Biology Reports. 2018; 45: 1469–1478.

[54] Hay P, Czeizel AE. Asymptomatic trichomonas and candida colonization and pregnancy outcome. Best Practice & Research. Clinical Obstetrics & Gynaecology. 2007; 21: 403–409.

[55] Schuster HJ, de Jonghe BA, Limpens J, Budding AE, Painter RC. Asymptomatic vaginal Candida colonization and adverse pregnancy outcomes including preterm birth: a systematic review and meta-analysis. American Journal of Obstetrics & Gynecology MFM. 2020; 2: 100163.

[56] Benchimol M, de Andrade Rosa I, da Silva Fontes R, Burla Dias AJ. Trichomonas adhere and phagocytose sperm cells: adhesion seems to be a prominent stage during interaction. Parasitology Research. 2008; 102: 597–604.

[57] Roh J, Lim YS, Seo MY, Choi Y, Ryu JS. The secretory products of Trichomonas vaginalis decrease fertilizing capacity of mice sperm *in vitro*. Asian Journal of Andrology. 2015; 17: 319–323.

[58] Zhang Z, Li Y, Lu H, Li D, Zhang R, Xie X, *et al*. A systematic review of the correlation between Trichomonas vaginalis infection and infertility. Acta Tropica. 2022; 236: 106693.

[59] Dhumal SS, Naik P, Dakshinamurthy S, Sullia K. Semen pH and

its correlation with motility and count-a study in subfertile men. JBRA Assisted Reproduction. 2021; 25: 172–175.

[60] Linderblood KL, Wilson WA, Brittingham A. Glycogen accumulation in trichomonas is driven by the availability of extracellular glucose. The Journal of Parasitology. 2021; 107: 514–518.

[61] Huang KY, Ong SC, Wu CC, Hsu CW, Lin HC, Fang YK, *et al*. Metabolic reprogramming of hydrogenosomal amino acids in Trichomonas vaginalis under glucose restriction. Journal of Microbiology, Immunology, and Infection. 2019; 52: 630–637.

[62] Huang KY, Chen YYM, Fang YK, Cheng WH, Cheng CC, Chen YC, *et al*. Adaptive responses to glucose restriction enhance cell survival, antioxidant capability, and autophagy of the protozoan parasite Trichomonas vaginalis. Biochimica et Biophysica Acta. 2014; 1840: 53–64.

[63] Numata S, McDermott JP, Sanchez G, Mitra A, Blanco G. The sodium-glucose cotransporter isoform 1 (SGLT-1) is important for sperm energetics, motility, and fertility. Biology of Reproduction. 2022; 106: 1206–1217.

[64] Kranjcić-Zec I, Dzamić A, Mitrović S, Arsić-Arsenijević V, Radonjić I. The role of parasites and fungi in secondary infertility. Medicinski Pregled. 2004; 57: 30–32.

[65] Keum J, Roh J, Ryu JS, Ryu KY. Excretory-Secretory Products of Trichomonas vaginalis Cause Apoptosis in Mouse Sperm *in Vitro*. The Korean Journal of Parasitology. 2022; 60: 357–360.