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The relationship between genitourinary microorganisms and oxidative stress, sperm DNA fragmentation and semen parameters in infertile men

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Abstract

An imbalance in the genitourinary microbiome is emerging as a contributing factor to male infertility. The purpose of this study was to determine whether there is an association between genitourinary microorganisms and seminal oxidative stress, sperm DNA fragmentation and semen parameters. It included 770 men attending for diagnostic testing for subfertility. Genitourinary microorganisms were identified in 43.0% men; 20.1% had microorganisms in semen; 18.7% in urine; and 5.8% had microorganisms in urine and semen. *Enterococcus faecalis* was the most prevalent organism in semen (22.0% samples; 61.5% organisms) with *Ureaplasma* spp. (16.9% samples; 53.3% organisms) and *Gardnerella vaginalis* (11.4% samples; 37.4% organisms) most prevalent in urine. Semen parameters were unaffected by microorganisms ($p > 0.05$). Seminal ROS were significantly higher in men with microorganisms compared to those without ($p < 0.001$), particularly when present in both urine and semen ($p < 0.01$). Microorganisms were associated with significantly higher DNA fragmentation, irrespective of whether they were in semen or urine ($p < 0.001$). An imbalance in the genitourinary microbiome is associated with DNA damage and oxidative stress which may have considerable consequences for achieving an ongoing pregnancy. This highlights the need for incorporating genitourinary microorganism screening for all men as part of diagnostic evaluation prior to undergoing treatment for infertility.

KEYWORDS
DNA fragmentation, genitourinary microorganisms, infertility, oxidative stress, sperm

1 | INTRODUCTION

Infertility is a global health issue affecting approximately 15% of couples (Mascarenhas et al., 2012) with a male factor contributing to almost 50% cases. Male infertility is a highly complex, multifactorial disorder with a variety of causes (Barratt et al., 2017; Matzuk & Lamb, 2008; Nieschlag & Behre, 2001; Tremellen, 2008). For some time, it has been recognized that a diverse vaginal microbiome has a negative association with fertility. More recently, it has become apparent that the semen hosts a widely variable microbiota that not only plays a role in male infertility but can also influence the female microbiome following sexual intercourse (Farahani et al., 2021; Koedooder et al., 2019; Osadchiy et al., 2020). Interestingly, as with the vaginal microbiome, the more diverse semen microbiomes are

more likely to be associated with infertility (Koedooder et al., 2019). Genitourinary infection is an attributable cause in 9.3%–15% cases of male infertility (Nieschlag & Behre, 2001; Pellati et al., 2008), resulting in urethritis, epididymitis, epididymo-orchitis or prostatitis (Naber et al., 2001; Nieschlag & Behre, 2001; Solomon & Henkel, 2017). Men are not routinely screened for genitourinary microorganisms (GM) which may be asymptomatic. Consequently, underlying microorganisms may remain undiagnosed. Almost 30% cases of male infertility remain unexplained (Nieschlag & Behre, 2001) so it is plausible that asymptomatic GM may contribute.

Considerable evidence reveals oxidative stress (OS) plays a fundamental role in the pathology of male infertility, particularly in idiopathic infertility (Agarwal et al., 2019; Tremellen, 2008). OS results from an imbalance between reactive oxygen species (ROS) production and generation of antioxidants leading to supraphysiological ROS levels (Tremellen, 2008). Thirty to 80% of infertile men have elevated seminal ROS (Agarwal et al., 2019). ROS are generated intrinsically from sperm (Bennetts et al., 2008) or extrinsically by activated leucocytes (Aitken & Koppers, 2011; Keck et al., 1998; Tremellen, 2008). Redox homeostasis is controlled by both endogenous and exogenous antioxidants (Dias et al., 2020; Martin-Hidalgo et al., 2019). Consequently, a reduction in seminal antioxidant capacity results in OS (Aitken & De Iuliis, 2010; Jones et al., 1978). OS interferes with a multitude of metabolic processes with significant effects on sperm function. ROS impair sperm mitochondrial and plasma membrane integrity via peroxidative damage (Jones et al., 1978, 1979) simultaneously reducing sperm motility (Alvarez et al 2002; Koppers et al., 2008). In addition, membrane lipid peroxidation produces mutagenic by-products with potentially serious consequences for the offspring (Aitken et al., 2016; Moazamian et al., 2015). OS also interferes with capacitation and fertilization and is linked with poor embryo development (Agarwal et al., 2019; Aitken, 2017; Nieschlag & Behre, 2001). Sperm DNA is a prime target for oxidative damage, causing single- and double-stranded DNA breaks. DNA fragmentation is significantly elevated in infertile men (Oleszczuk et al., 2013; Santi et al., 2018) and is associated with failure of fertilization and embryogenesis both from natural conception and assisted reproductive technology (ART) (Benchaib et al., 2007; González-Marín et al., 2012; Jin et al., 2015; Osman et al., 2015; Simon et al., 2010), as well as miscarriage (Simon & Carrell, 2013). Furthermore, sperm DNA fragmentation is linked with an increased risk of gene mutations, congenital malformations and childhood diseases (Aitken & Curry, 2011; Bisht & Dada, 2017; Sakkas & Alvarez, 2010). Recently, ESHRE (Bender Atik et al., 2018), EAU (Minhas et al., 2021) and AUA/ASRM (Schlegel et al., 2021) guidelines acknowledged the potential contribution of sperm DNA fragmentation towards male infertility.

Evidence suggests that while some microorganisms such as *Chlamydia trachomatis* (Hughes & Field, 2015) may not necessarily cause any symptoms, nevertheless, they may have detrimental effects on fertility and pregnancy (Lundy et al., 2020; Solomon & Henkel, 2017). Furthermore, changes in the microbiome of the male reproductive tract are associated with poor fertilization and embryo development (Montagut et al., 1991), negative outcomes for ART (Ricci et al., 2018; Zeyad et al., 2018), complications during pregnancy and birth (Kalinderi et al., 2018) as well as recurrent miscarriage and adverse perinatal outcomes (Cohen et al., 2019; Howley et al., 2018). Bacterial microorganisms and the host inflammatory reaction trigger an overwhelming release of ROS in the local environment (Agarwal et al., 2018; Micheli et al., 2016). Therefore, GM could potentially lead to infertility as a result of ROS generation and subsequent DNA damage. Until now, studies investigating the relationship between GM, OS and DNA damage have been limited and their inter-relationship have not been explored in depth. The aim of this study was to determine the prevalence of GM in a cohort of infertile men and determine any association with semen parameters, leucocytes, generation of OS and sperm DNA damage. There is some controversy concerning the clinical value of available tests for assessing sperm DNA damage, although most are predictive of infertility (Javed et al., 2019; Ribas-Maynou et al., 2013). While SCSA and Comet assay are reliable and validated tests, results often differ primarily because they are measuring different aspects of DNA damage. Comparisons between tests are difficult to interpret as they are not consistently performed on the same samples and threshold values differ between laboratories performing the assay (Evenson, 2016; Ribas-Maynou et al., 2013). Furthermore, outcome studies are not always controlled for female factors. Thus, sperm DFI levels were investigated in infertile men with microorganisms using both SCSA and Comet methods relying on previously validated threshold values. Results were compared to infertile men without GM as controls.

2 | MATERIALS AND METHODS

This retrospective study involved audit and data analysis of anonymized patient results from 770 men attending for diagnostic tests and investigation of infertility between 1 January 2015 and 31 December 2019. The study was approved by the Faculty of Sciences Research Ethics Advisory Group for Human Participants at the University of Kent (ID number 0651718).

2.1 | Semen and urine sample collection

Patients were provided with strict instructions on sample production to mitigate against contamination (World Health Organization, 2010). Urine samples collected for PCR were obtained from the first catch. Mid-stream samples were collected using a separate sterile container for standard culture. Semen samples were collected via masturbation into a sterile container. Sexual abstinence prior to testing was 2–5 days. Samples for semen analysis and DNA fragmentation were processed within 1 h of ejaculation. OS measurement was routinely performed 20 min postejaculation. Samples where patients reported noncompliance or fever within 12 weeks prior to testing were excluded. Results from the first semen sample were recorded for patients with multiple semen analyses. All diagnostic

testing was carried out at a UKAS accredited pathology laboratory in London, UK (The Doctors Laboratory).

2.2 | Semen parameters

Semen parameters were assessed according to WHO 2010 guidelines (World Health Organization, 2010). Count was assessed on fixed sperm using an improved Neubauer chamber. Papanicolaou staining was utilized for morphological analysis according to Kruger strict criteria. Vitality was measured by dye-exclusion with 0.5% Eosin-Y in 0.9% NaCl. Peroxidase staining, in conjunction with cytological differential staining, identified seminal leucocytes. Leucocytospermia was defined when leucocyte concentration was $\geq 1 \times 10^6$/ml semen (World Health Organization, 2010).

2.3 | Screening for microorganisms

Genitourinary microorganisms were determined from culture and PCR analysis. Ten microlitres of semen or urine was applied to each of the following culture plates and spread to visualize individual colony-forming units (CFUs): Columbia CAP Agar/CLED Medium Biplate; PB1248E, incubated up to 36 h aerobically at 35°C; Columbia Agar with Chocolate Horse Blood; PB0124E, incubated up to 36 h in 7% CO_2 at 35°C; Lysed GC selective agar; PB1205A, incubated for 40 h in 7% CO_2 at 35°C; Brilliance Candida Agar; PO1034A, incubated for 48 h aerobically at 35°C; A.R.I.A. Medium with 5% Horse Blood/A.R.I.A. Medium with 5% Horse Blood and Neomycin Biplate; PB1260E, incubated for 48 h anaerobically at 35°C. All plates were supplied by Oxoid Ltd. All colonies were identified by Maldi-Tof.

To distinguish between contamination and prevalence of microorganisms, bacterial data were only included if there were $>10^3$ CFUs/ml (Koeijers et al., 2010; Lipsky et al., 1987). PCR was used for urine and semen samples to detect seven sexually transmissible microorganisms (STIs): *Neisseria gonorrhoea*, *Chlamydia trachomatis*, *Mycoplasma genitalium*, *Ureaplasma species*, *Gardnerella vaginalis*, Trichomonas, Herpes simplex virus (HSV) I and II. Fast Track Diagnostics STD9 (Cat no. FTD 52.1) and Vesicular Rash (Cat. no. FTD-7–64) kits (Siemens Healthineers) based on real-time PCR amplification of nucleic acid from bacteria, viruses and protozoa were used in conjunction with QIA symphony and Rotor-gene Q to identify microorganisms according to the manufacturer's guidelines. Specific pathogen sequences were detected by an increase in fluorescence from the relevant dual-labelled probe and was reported as a cycle threshold value by the real-time thermal cycler.

2.4 | DNA fragmentation

Comet (Simon & Carrell, 2013) or SCSA (Evenson et al., 1999) were performed by Examen and SCSA Diagnostic laboratories, respectively. DNA fragmentation index (DFI) and HDS were measured by SCSA. HDS is the percentage sperm with high levels of green fluorescence, which represents the proportion of immature spermatozoa with incomplete chromatin condensation (Evenson, 2016). The average DNA fragmentation score was measured by Comet.

2.5 | Semen ROS

Reactive oxygen species was determined using a chemiluminescence assay using a single tube luminometer described previously (Agarwal et al., 2016). Results were adjusted for sperm concentration and expressed as relative light units (RLU)/s/10^6 sperm.

2.6 | Statistical analysis

The dataset was imported from an electronic database into SPSS 26.0 software for Windows (SPSS Inc) for analysis. All parameters had non-normally distributed continuous data with skewed distributions, confirmed mathematically using the Kolmogorov–Smirnov test. All numerical values, unless otherwise stated, were reported using the median (inter-quartile range). Data were analysed using nonparametric statistics, such as Kruskal–Wallis test. Post hoc testing with Dunn's pairwise analyses was used to assess significant differences in semen parameters between males with and without GM. Chi-squared tests compared proportions between two or more categorical groups. Relationships were determined using Spearman's correlation. A multiple regression analysis was performed with 'best fit' regression as the combination of variables that best predicts the infected versus noninfected samples. Differences were considered statistically significant when $p < 0.05$. Bonferroni correction was applied to *p*-values for multiple comparisons to reduce type one errors. All graphs were generated using GraphPad 50 (GraphPad Software, Inc).

3 | RESULTS

3.1 | Distribution of genitourinary tract microorganisms in semen and urine

Of the 770 patients recruited for the study, semen screening was performed for 740 men (595 for culture; 532 for PCR) and urine screening for 748 men (677 for culture; 657 for PCR). Four groups of patients were identified. Group 1 had no bacteria in urine or semen (439/770; 57.0%). In total, GM were identified in 43.0% (331/770) men. Group 2 had at least one organism present in semen (149/740; 20.1%), Group 3 had at least one organism present in urine (140/748; 18.7%) while Group 4 had microorganisms in both semen and urine (42/726; 5.8%). Of the 331 patients with GM, semen or urine microorganisms were present in 45.0% ($n = 149$, Group 2) and 42.3% ($n = 140$, Group 3) respectively. The remaining 12.7% ($n = 42$, Group 4) had microorganisms in both semen and urine. The median age of

the study population was 37 years (IQR: 34–41). There was no significant difference in age distribution among study groups (Group1: 37 [IQR: 34–41]; Group2: 39 [IQR: 35–42]; Group3: 37 [IQR: 34–41]; Group4: 36 [IQR: 33–40; $p = 0.054$]). The median duration for attempting to conceive was 2 (IQR: 1–3) years and did not differ between groups (Group1: 2 [IQR: 1–3]; Group2: 2 [IQR: 1–3]; Group3: 1.5 [IQR: 1–3]; Group4: 2.5 [IQR: 1–3; $p > 0.05$]). Other demographic information such as ethnicity were not analysed as a significant proportion of this data was unattainable.

Table 1 shows the distribution of microorganisms in semen and urine of subfertile men. Using a PCR screen for seven different organisms combined with routine culture methods, a total of 28 different organisms were identified. Semen microorganisms were identified in 33.4% of all samples that were cultured (199/595) but in only 2.6% of samples that were screened by PCR (14/532). On the other hand, urine microorganisms were identified in only 3.1% (21/677) cultures but in 29.4% (193/657) samples screened by PCR. Most men with GM had a single species of organism identified in either urine or semen. In some cases, more than one species of microorganism was identified. *Enterococcus faecalis* was present in 21.8% of all semen cultures and was the most prevalent microorganism identified using this method (Table 1). Apart from *Enterococcus faecalis*, *Candida sp.* and *Citrobacter koseri* were the next most common organisms in semen culture (Table 1), but were only detected in 1.7% and 1.2% of all semen cultures respectively. In contrast, *Ureaplasma* species was the most frequent microorganism detected in urine followed by

TABLE 1 Frequency of microorganisms in semen and urine of subfertile men

	Semen		Urine	
	Number of samples with organisms	% Total organisms	Number of samples with organisms	% Total organisms
Enterococcus faecalis	131	61.5	2	0.9
Ureaplasma species	12	5.6	114	53.3
Gardnerella vaginalis	11	5.2	80	37.4
Candida species	10	4.7	0	0.0
E. coli	3	1.4	5	2.3
Citrobacter koseri	7	3.3	1	0.5
Chlamydia trachomatis	0	0.0	0	0.0
Neisseria gonorrhoeae	0	0.0	1	0.5
Mycoplasma genitalium	0	0.0	5	2.3
Mycoplasma hominis	5	2.3	0	0.0
Morganella morganii	3	1.4	1	0.5
Proteus mirabilis	2	0.9	1	0.5
Aerococcus urinae	3	1.4	0	0.0
Corynebacterium species	1	0.5	0	0.0
Streptococcus mitis	1	0.5	0	0.0
Staphylococcus epidermidis	5	2.3	0	0.0
Streptococcus milleri	2	0.9	0	0.0
Streptococcus agalactiae	1	0.5	0	0.0
Streptococcus anginosus	3	1.4	0	0.0
Beta-haemolytic streptococcus	3	1.4	1	0.5
Actinobacillus species	1	0.5	0	0.0
Streptococcus gallolyticus	1	0.5	0	0.0
Peptostreptococcus species	2	0.9	0	0.0
Serratia marcescens	1	0.5	0	0.0
Staphylococcus oralis	1	0.5	0	0.0
Staphylococcus haemolyticus	4	1.9	0	0.0
Klebsiella variicola	0	0.0	1	0.5
Klebsiella pneumoniae	0	0.0	1	0.5
Herpes simplex Type 1/2	0	0.0	0	0.0
Trichomonas vaginalis	0	0.0	1	0.5
TOTAL	213	100	214	100

Gardnerella vaginalis (Table 1). Ureaplasma was present in 16.9% of all urine samples assessed by PCR screening, while Gardnerella was present in 11.4% of all urine samples assessed by PCR. Of the 532 semen samples screened for microorganisms by PCR, only 0.9% contained *Ureaplasma* species and only 1.7% contained *Gardnerella vaginalis*. The most common microorganism found in urine culture was *E. coli*, but this was only detected in less than 1% of all samples that were cultured.

3.2 | Effects of genitourinary tract microorganisms on semen parameters

Apart from seminal leucocytes, there was no significant difference in semen parameters between study groups. Using a standard *t*-test to compare the difference between infected versus noninfected groups for each individual infection, there was only a significant difference for the following variables: urine *Ureaplasma* spp., tail defects $p = 0.0068$; urine *Gardnerella vaginalis*, midpiece defects $p = 0.0210$; semen *Enterococcus faecalis*, abnormal morphology $p = 0.139$; tail defects $p = 0.0154$ and nonprogressive motility $p = 0.0034$. Association of bacteria with semen parameters was also investigated using multiple regression analysis and only nonprogressive motility was significantly associated with the presence of bacteria (semen *Enterococcus spp.* $p = 0.003$). Overall, the median value for all semen parameters in all study groups were within reference range (World Health Organization, 2010) with the exception of morphology which showed a high level of abnormal forms in all groups including the group without GM (Group1; Table 2). Viscosity was normal in all samples and antisperm antibodies were undetected. pH remained consistent at 8.2 in all groups and vitality was unaffected by GM, remaining within reference range (>58%; World Health Organization, 2010).

3.3 | Peroxidase-positive leucocytes, reactive oxygen species and sperm DNA damage

The peroxidase-positive leucocyte concentration remained within the reference range for all patient groups (<1 million/ml; World Health Organization, 2010) (Figure 1A), although patients with urine microorganisms (Group 3) had significantly higher concentrations compared to samples without GM (Group 1; $p < 0.001$). In contrast, leucocyte concentrations in other infected groups were not significantly different from those without microorganisms ($p > 0.05$). Leucocyte concentrations positively correlated with ROS levels ($r_s = 0.263$; $p < 0.001$). Seminal ROS levels in men without GM (Group1: 2.85 [0.70–12.35] RLU/s/10^6 sperm; Figure 1b) were well below the upper reference limit of 13.8 RLU/s/10^6 sperm, determined from ROC analysis of 854 samples with 86% sensitivity and 86% specificity. The semen or urine microorganisms group had ROS levels 5.2 and 4.6 times higher than Group 1 respectively (Group 2:14.70 [5.93–43.03]; Group3:12.95 [5.80–35.4] RLU/s/10^6 sperm; $p < 0.001$). Using multiple regression analysis, ROS was significantly associated with the presence of *Ureaplasma* spp. ($p < 0.001$) and *Gardnerella vaginalis* ($p < 0.001$) in urine and *Enterococcus spp.* ($p < 0.001$) in semen. Patients with both semen and urine microorganisms (Group 4) had the highest ROS levels with at least a 10-fold increase in ROS compared to patients without microorganisms (29.50 [0.90–241.20] versus 2.85 [0.70–12.35] RLU/s/10^6 sperm; $p < 0.01$) and was significantly above the normal range. Semen (odds ratio [OR] 3.84; 95% CI 2.24–6.60; $p < 0.001$) or urine (OR 3.21; 95% CI 1.90–5.44; $p < 0.001$) microorganisms significantly increased the odds of having elevated ROS levels. Therefore, ROS was the most affected semen parameter in the presence of GM among those analysed (Figure 1b). ROS negatively correlated with per cent total motility ($r_s = -0.196$; $p < 0.001$) and total motile sperm count ($r_s = -0.346$; $p < 0.001$). Moreover, an association was observed between ROS and DFI as measured by SCSA ($r_s = 0.182$; $p < 0.05$). However, no correlation was found between ROS and average Comet scores ($p > 0.05$). When sperm DNA damage was measured with SCSA (Figure 1c), sperm DFI in samples with semen or urine microorganisms were significantly higher than those without GM (Figure 1c SCSA: Group 1: 15.0 [10.0–23.0]%; Group 2: 20.5 [17.0–38.8]%; Group 3: 28.5 [16.0–40.0]%; Group4: 36.5 [24.8–49.0]; $p < 0.001$). The same was true for sperm DNA damage when measured by Comet (Figure 1d Comet: Group1: 32.0 (28.0–41.5)%; Group2: 38 (32.0–47.8)%; Group3: 39.5 (34.8–47.3)%; Group4: 44.5 (41.3–52.3)%; $p < 0.001$). Patients with both semen and urine microorganisms (Group 4) had the highest DFI, whereas patients without GM had the lowest, irrespective of how DNA damage was measured ($p < 0.05$). Semen (OR 3.18; 95% CI 1.22–8.28; $p < 0.05$) or urine (OR 5.33; 95% CI 2.00–14.25; $p < 0.001$) microorganisms increased the odds of having an elevated DFI. Although DFI was increased in all samples in the presence of microorganisms, only those with urinary microorganisms (with or without semen microorganisms) had DFI levels above the reference threshold limit when measured by SCSA. The results from multiple regression analysis demonstrate the most consistent associations between infected samples were with the DNA fragmentation scores (either DFI or COMET). COMET was significantly associated with the presence of *Ureaplasma* spp. ($p < 0.001$) in urine, while DFI was significantly associated with the presence of *Gardnerella vaginalis* ($p < 0.001$) in urine and *Enterococcus spp.* ($p = 0.003$) in semen. There was also an association between HDS and the presence of microorganisms. HDS was higher in Group 3 (21.0 [18.0–26.0]) and Group 4 (25.0 [16.0–30.5]) compared to Group 1 (11.0 [6.0–16.0]). A significant difference in HDS was only found between Group 3 with urine microorganisms and Group 1 without ($p < 0.05$). HDS levels in samples with semen microorganisms (Group 2: 11.0 [6.3–19.8]) were similar to those without (Group 1: (11.0 [6.0–16.0]). In contrast all patient groups including Group 1 had average Comet scores higher than the reference range.

4 | DISCUSSION

To our knowledge, this is the first and largest study to date to show an association of semen and urine microorganisms with both ROS

TABLE 2 Differences in standard semen parameters between patient study groups

Patient GROUP	1	2	3	4	*p* value
Number	439	149	140	42	
Volume (ml)	3.40 (2.60–4.40)	3.30 (2.10–4.40)	3.60 (2.50–4.65)	3.5 (2.40–4.60)	0.392
Total motility (%)	58.00 (37.00–65.00)	56.00 (37.50–66.50)	58.00 (33.00–67.00)	60.00 (39.25–68.00)	0.830
Progressive motility (%)	48.00 (27.00–59.00)	44.00 (19.50–59.00)	47.50 (21.50–60.25)	49.00 (21.25–61.00)	0.875
Nonprogressive motility (%)	7.00 (4.00–11.00)	8.00 (4.00–12.00)	7.00 (3.75–10.00)	6.00 (5.00–10.00)	0.494
Immotile sperm (%)	40.00 (33.00–57.50)	43.00 (33.00–59.00)	40.00 (32.00–61.50)	39.00 (30.00–60.00)	0.903
Total motile sperm count ($\times 10^6$)	56.00 (22.00–117.00)	45.00 (14.00–112.00)	82.00 (7.60–146.50)	101.00 (28.00–131.00)	0.570
Sperm count ($\times 10^6$/ml)	22.00 (6.65–54.00)	25.00 (7.90–55.00)	19.00 (5.00–47.00)	28.00 (9.40–60.00)	0.453
Total sperm count ($\times 10^6$)	70.00 (22.75–182.00)	78.00 (25.00–155.95)	65.50 (17.00–173.50)	83.50 (24.00–236.75)	0.738
Normal morphology (%)	2.00 (1.00–5.00)	2.00 (1.00–3.75)	2.00 (1.00–6.00)	2.00 (0.50–6.00)	0.560
Teratozoospermia index (TZI)	1.38 (1.30–1.47)	1.37 (1.31–1.46)	1.39 (1.31–1.47)	1.40 (1.32–1.50)	0.853
Leucocyte count (10^6/ml)	0.10 (0.10–0.40)	0.20 (0.10–0.40)	0.20 (0.10–0.50)	0.15 (0.10–0.40)	0.001
ROS (RLU/second /10^6 sperm)	2.85 (0.70–12.35)	14.70 (5.93–43.03)	12.95 (5.80–35.40)	29.50 (0.90–241.20)	<0.001
DFI (%)	15.00 (10.00–23.00)	20.50 (17.00–38.75)	28.50 (16.00–40.00)	36.50 (24.75–49.00)	<0.001
Average comet score (%)	32.00 (28.00–41.50)	38.00 (32.00–47.75)	39.50 (34.75–47.25)	44.50 (41.25–52.25)	<0.001

and sperm genetic integrity. GM were detected in 43% infertile men and were associated with elevated ROS and sperm DNA fragmentation, measured either by Comet or SCSA. Standard semen parameters were minimally affected by GM and were similar across patient cohorts. While this concurs with previous studies (Filipiak et al., 2015; Hillier et al., 1990; Qing et al., 2017), effects of bacterial microorganisms on semen parameters remain controversial. On the contrary, a recent meta-analysis and systematic review of the impact of the semen microbiome on male infertility, involving 55 observational studies with 51,299 men, revealed GM had a negative impact overall on sperm count, progressive motility and sperm DNA integrity (Farahani et al., 2021).

The incidence of semen microorganisms in infertile men (20.1%) was similar to previous reports (21%–35.3%) (Moretti et al., 2009; Ricci et al., 2018; Villanueva-Diaz et al., 1999; Vilvanathan et al., 2016; Zeyad et al., 2018) with *Enterococcus faecalis* as the most abundant seminal microorganism. In contrast, a large Italian study found *Ureaplasma urealyticum* was the most common seminal microorganism (Boeri et al., 2020), whereas *Ureaplasma* spp. was the most prevalent single organism in urine in this study, followed closely by *Gardnerella*. The incidence of urine microorganisms in this study was considerably lower to that previously reported by Qing et al. (2017) although the incidence of *Ureaplasma* spp. among samples with GM was similar. Generally, the proportion of Ureaplasma microorganisms among infertile men is variable, ranging between 5% and 42% (Agarwal et al., 2018). While *E. coli* was detected infrequently in semen, a previous study demonstrated it is the second most prevalent seminal microorganism in infertile men (24%) (Villanueva-Diaz et al., 1999). However, it was the most prevalent microorganism detected by urine culture in this study. Globally, *E. coli* is the most prevalent urinary tract microorganism, while *Ureaplasma* also has a high prevalence (Solomon & Henkel, 2017).

The differences in prevalence of GM and their effects on semen parameters across studies may depend upon patient cohort, type and concentration of organisms present as well as the balance of organisms in the microbiome (Lundy et al., 2020; Oghbaei et al., 2020). The heterogeneity and availability of pathogen detection methods must also be considered. Urine microorganisms were most likely detected using PCR rather than culture. PCR analysis is only offered in specialized laboratories, hence GM may be missed in asymptomatic patients which may have implications regarding the management of infertility. Studies on the microbiome are often limited by the methods of analysis, particularly with regard to the sensitivity of detection and the number of microorganisms that can be detected. A limitation of this study is that the PCR methodology does not allow for quantitative analysis of these microorganisms which may well have specific effects on individual sperm parameters, including DNA damage and seminal OS. A more accurate representation of the seminal and urinary microbiome could be provided by genomic sequencing. Nonetheless, previous studies have detected similar species and proportions of microorganisms in semen and urine overall.

Previous studies associated positive semen cultures with significantly reduced sperm motility (Micheli et al., 2016; Moretti et al., 2009; Ricci et al., 2018) possibly due to an elevation in OS. In this study, a significant negative correlation between sperm motility and ROS was identified, confirming our previous observations (Homa et al., 2019). As a result, microorganisms may exert detrimental effects on fertility primarily through generation of OS and consequent sperm DNA damage (Agarwal et al., 2014). The data presented here lends support for an association between GM and OS and damage to sperm chromatin integrity (Agarwal et al., 2018; Gallegos et al., 2008;

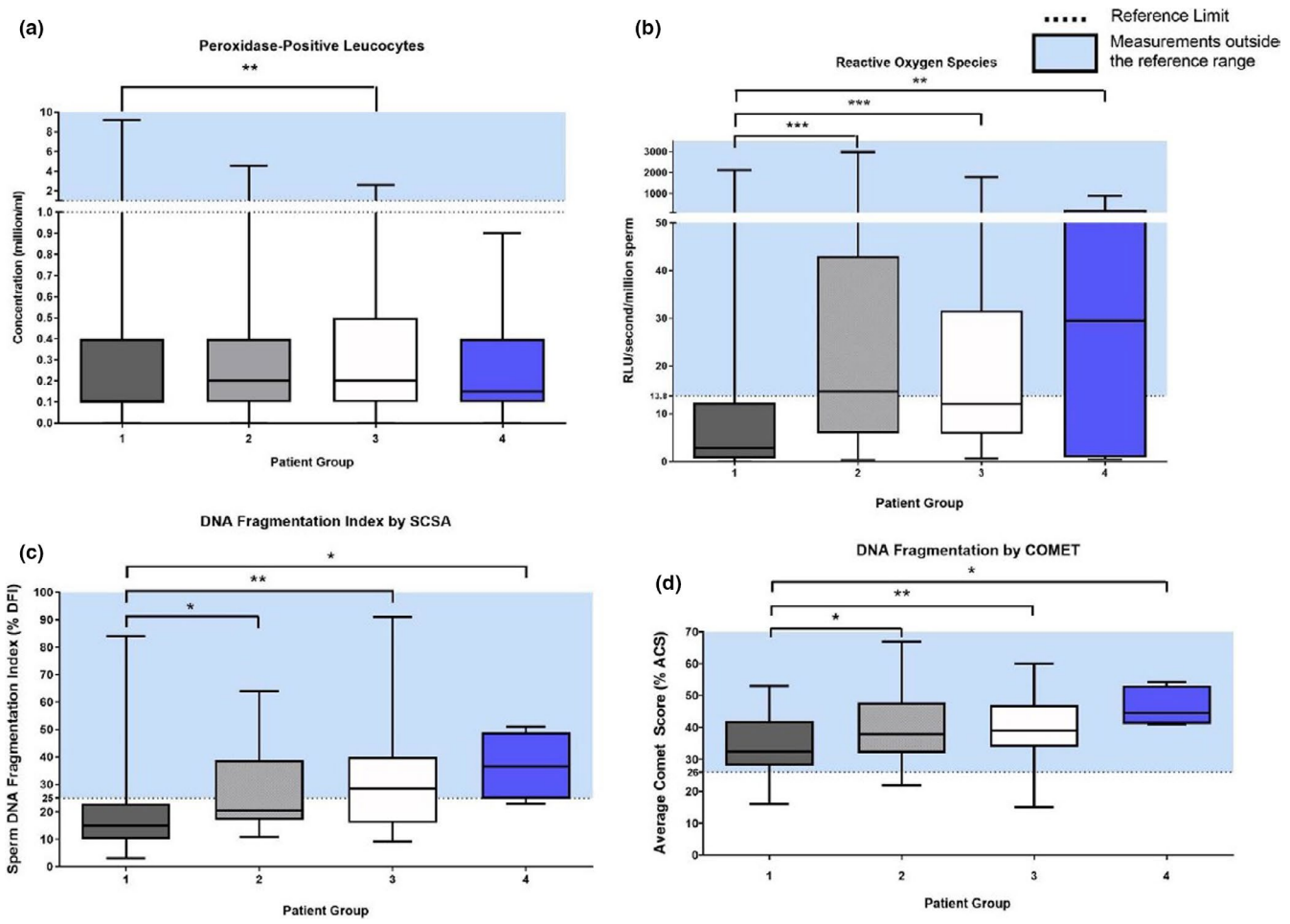

FIGURE 1 Comparison of sperm DNA fragmentation, oxidative stress and leucocyte levels in semen of infertile men with and without GM. Box-whisker plots displaying leucocyte concentrations (a), ROS levels (b), DFI (c) and COMET scores (d); Patients with no genitourinary microorganisms (Group 1: $n = 439$), semen microorganisms (Group 2: $n = 149$), urine microorganisms (Group 3: $n = 140$) or both semen and urine microorganisms (Group 4: $n = 42$). The dotted line indicates the reference limit (Leucocytes: <1 million/ml; ROS: <13.8 RLU/s/10^6 sperm; DFI by SCSA: <25%; DFI by COMET: <26%) and the shaded area represents the area where values are outside the reference range. The box lies between the first and third quartiles, thus covering the middle 50% of all data values, with the median represented by the middle line. Whiskers extend from minimum to maximum values. Kruskal–Wallis test: $p < 0.01$ for a, $p < 0.001$ for b, c and d. Dunn's post hoc analysis (with significance values adjusted by the Bonferroni correction for multiple tests): *, ** and *** indicate $p < 0.05$, 0.01 and 0.001 respectively. Abbreviations: DNA, deoxyribonucleic acid; RLU, relative light units; SCSA, sperm chromatin structure assay

Oghbaei et al., 2020; Potts & Pasqualotto, 2003; Qing et al., 2017; Reichart et al., 2000). In contrast, a previous study failed to find any effect of *Chlamydia trachomatis*, *Ureaplasma* or *Mycoplasma spp*. on DNA fragmentation measured by SCSA (Rybar et al., 2012). Bacterial microorganisms of the genital tract not only generate high ROS levels but also deplete antioxidant capacity (Micheli et al., 2016) resulting in OS. Additionally, leucocytes, a significant source of exogenous ROS (Aitken et al., 1994), will migrate into the site of infection. However, seminal leucocytes remained below the World Health Organization (2010) threshold in all GM groups, even though ROS and sperm DNA damage were significantly increased, suggesting ROS generation is derived directly from bacterial activity. Similarly, Gallegos et al. (2008) observed that patients with GM had increased sperm DNA damage despite the absence of leucocytospermia. The finding of low levels of leucocytes in infected samples is not altogether unexpected as previous studies indicated leucocyte concentrations are poorly predictive of seminal microorganisms (Chen et al., 2013; Hillier et al., 1990). Furthermore, OS associated with chronic prostatitis is independent of leucocytospermia (Nickel et al., 2003; Pasqualotto et al., 2000). Leucocytospermia may be dependent on sampling technique as prostatic massage may be required prior to ejaculation to express leucocytes into seminal fluid (Ludwig et al., 2003).

One of the most detrimental effects of OS is oxidation of sperm DNA (Aitken & De Iuliis, 2010; Aitken & Koppers, 2011; Wright et al., 2014). Men with GM demonstrated significantly elevated ROS in association with elevated SCSA and Comet scores. In a prospective study, high Comet scores were associated with an eight-fold increase in the risk of infertility (Simon et al., 2011). Furthermore, males with high DFI and Comet scores have reduced fertilization rates, embryo quality and pregnancy outcomes from IVF (Haddock et al., 2021;

Simon et al., 2011; Virro et al., 2004) and ICSI (Simon et al., 2010; Virro et al., 2004). SCSA and Comet are also valuable in predicting miscarriage (Evenson et al., 1999; Yifu et al., 2020) and live birth rates (Osman et al., 2015). Hence, via generation of OS and DNA damage, male GM may play a much wider role in infertility than previously thought.

Both SCSA and alkaline Comet tests were associated with the highest increases in sperm DNA fragmentation when microorganisms were present in both genital and urinary compartments. Comet scores were above the threshold for all patient groups including those without microorganisms, although only urinary tract microorganisms caused an elevation in DFI above the threshold limit when measured by SCSA. While the trends in results using the SCSA and Comet test are similar, other studies support our observations that Comet scores are consistently higher than those measured by SCSA (Javed et al., 2019). The differences in the scale of these observations may be because different assays focus on different molecular aspects of sperm DNA damage. Consequently, Comet may have a higher sensitivity than SCSA. In addition, HDS levels were approximately doubled in the presence of urine microorganisms but were not increased if microorganisms were identified in semen alone. HDS is a biomarker for abnormal chromatin and a recent study demonstrated an association between.

Staphylococcus spp., *E. coli*, *Enterococcus faecalis and Streptococcus agalactiae* in semen and abnormal chromatin condensation and protamine P1/P2 ratios (Zeyad et al., 2018). Urine microorganisms may thus have consequences for ongoing pregnancy, particularly as HDS may be associated with an increased risk of spontaneous abortion (Lin et al., 2008).

It is clear that GM are associated with OS and DNA damage which in turn may explain the association of genital tract dysbiosis and infertility. The mechanism of action may well involve direct binding of the bacteria to the sperm plasma membrane as well as secretion of cytotoxins all of which may generate ROS resulting in considerable cellular and DNA damage (reviewed by Henkel et al., 2021; Oghbaei et al., 2020; Osadchiy et al., 2020). Many bacteria, including *Ureaplasma* spp., *Klebsiella pneumonia* and *E. coli* can bind to membrane glycoproteins or mannose receptors. *Ureaplasma* binding triggers ROS generation and subsequent membrane lipid peroxidation, causing DNA damage and disruption to the acrosomal membrane which has significant consequences for fertilization (Ma & Gao, 2017). Furthermore, *E. coli* interferes with fertilization by impairment of the acrosome reaction (El-Mulla et al., 1996). Adhesion of *E. coli* to sperm or exposure of sperm to its soluble factors leads to a decrease in mitochondrial membrane potential and an increase in apoptotic markers such as phosphatidylserine translocation (Schulz et al., 2010) while Chlamydia trachomatis is associated with both phosphatidylserine externalization and sperm DNA damage (Satta et al., 2006). Hemolyisn released from *Enterococci* (Qiang et al., 2007) and lipopolysaccharides from *E. coli* (Wolff et al., 1993) or Chlamydia (Eley et al., 2005) cause sperm immobilization and cell death, likely as a consequence of ROS production, release of cytochrome C and activation of caspases 3 and 9 (Said et al., 2004). This sequence of events leads to apoptosis which is intricately linked with DNA strand breaks (Agarwal & Said, 2005). An alternative mechanism for GM-induced sperm DNA damage may be the generation of a localized immune response producing proinflammatory cytokines and ROS which may disrupt spermatogenesis and have a negative effect on sperm motility and cause cell death (Henkel et al., 2021). Several bacterial species including *E. coli*, *Streptococcus oralis*, *Staphylococcus haemolyticus*, *Ureaplasma urealyticum and B. ureolyticus* are associated with local production of ROS and cytokines leading to plasma membrane lipid peroxidation and malondialdehyde production (Fraczek et al., 2007). This causes irreversible plasma membrane damage, DNA oxidation and impaired acrosome reaction and fertilization. Furthermore, *Enterococcus* is associated with inflammatory proteins in seminal plasma (Grande et al., 2018) and a reduction in semen parameters (Farahani et al., 2021; Ricci et al., 2018) and is a causative organism of prostatitis, while Ureaplasma and *E. coli* have also been associated with this inflammatory condition (Henkel et al., 2021). Finally, cross-reactive antibodies may be produced in response to seminal microorganisms (Shi et al., 2007; Witkin et al., 1995) which are a cause of immunological infertility.

While dysbiosis in both the male and female genital tract can affect reproductive health, it may also have a considerable impact on fertility and pregnancy outcome. *Ureaplasma urealyticum* in semen or in the female reproductive tract has deleterious consequences for embryo development as it decreases pregnancy rate following IVF (Montagut et al., 1991). IVF outcome was only 7.5% successful when *Ureaplasma* and/or *Enterococcus* was present in the GU tract (Ricci et al., 2018), most likely due to its effect on sperm DNA integrity and ROS production (Potts & Pasqualotto, 2003). More concerning, microorganisms of the female reproductive tract and particularly microorganisms causing bacterial vaginosis (BV) such as *Gardnerella vaginalis* and *Ureaplasma* spp., are consistently associated with infertility, recurrent miscarriage and preterm birth Koedooder et al., 2019; Giakoumelou et al., 2016; Kuon et al., 2017; Nelson et al., 2015). While women with *Gardnerella* and *Ureaplasma* may be symptomatic and are treated for these microorganisms, their partners who remain asymptomatic are not and consequently continue as a source for recurrent transmission of microorganisms to their partners. At present, screening men for GM is not routinely incorporated into clinical diagnostic protocols for male infertility, mainly because it is difficult to differentiate active microorganisms from commensal organisms in the genital tract. Furthermore, many GM are asymptomatic (Kiessling et al., 2008), hence patients do not actively seek treatment.

5 | CONCLUSIONS

This study presents evidence for the mechanistic role of the semen microbiome, whereby a prevalence of opportunistic bacteria such as *Ureaplasma* spp. and *Gardnerella vaginalis*, as well as pathobionts such as *Enterococcus faecalis* affect sperm function by triggering OS and DNA damage, irrespective of any effect on semen

parameters. This is likely to result in infertility with consequences for an ongoing pregnancy. Given the evidence presented here, it is suggested that GU screening of both partners should be incorporated into routine investigation of all couples unable to conceive or with a history of miscarriage, particularly prior to embarking on ART. Consideration should be given to the value of assessing both urine and semen for microorganisms in cases of unexplained infertility. The distribution of microorganisms differs between the urinary and genital tract, yet microorganisms in either compartment has a detrimental effect on OS and sperm genetic integrity. As PCR identifies organisms that cannot be identified using culture alone, it is recommended that both methods should be utilized. PCR screening in semen may be unnecessary as only 2.6% patients had microorganisms identified by this method, and only 0.75% patients screened by PCR in semen showed microorganisms that were not also identified in urine. Microorganisms can be managed with targeted antibiotic treatment in most cases and may optimize the chances of ongoing pregnancy, although care must be taken when using broad-spectrum antibiotics to avoid disturbing the seminal plasma microbiome.

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CONFLICT OF INTEREST

The authors declare no conflict of inerest

DATA AVAILABILITY STATEMENT

Data available on request due to privacy/ethical restrictions.

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