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ORIGINAL ARTICLE

Semen Analysis

Semen microbiota and cytokines of healthy and infertile men

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In this study, we determined the levels of cytokine secretory inhibitors and the microbiota biofilms of semen from healthy and infertile subjects. A total of 118 clinical bacterial isolates were isolated and tested. Cytokine secretory inhibitors were determined based on the difference in cytokine content between the control and experimental samples of cell-free supernatants of isolated microorganisms. Biofilm formation was studied by determining the adhesion of microorganisms to the surface of a 96-well sterile plate and expressed as the optical density at 630 nm (OD_{630}). Cell-free supernatants of *Staphylococcus* contained higher levels of secretory inhibitor of cytokines in conditionally healthy than in infertile patients. In contrast, in infertile men, the ability to reduce cytokine levels was more characteristic of *Enterococcus* and *Corynebacterium*. Seminal *Staphylococcus*, *Corynebacterium*, and *Enterococcus* isolated from infertile subjects showed a greater ability to form biofilms than the same bacteria isolated from healthy men. Further research is needed on this topic, since it is necessary to determine the relationships between decreased secretory inhibitors of cytokines, production of biofilms by bacteria in semen, and infertility. It is likely that the ability of microorganisms to change the concentration of cytokines and increase the level of biofilm formation in semen may be associated with minimal impairments of fertilizing ability, which are not detected using other methods.

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INTRODUCTION

Infertility, a condition different from various existing health-related issues involving psychological and social aspects, affects 10%–15% of couples in reproductive age.[1] Furthermore, infectious etiologies involving bacteria, viruses, fungi, and protozoa contribute to 15% of male factor infertility.[2,3]

Some studies have shown that the presence of bacteria in semen is common, including in healthy people.[4,5] The seminal plasma has a specific microbiota, and it can be postulated that the presence of a specific bacterial milieu may not be deleterious but necessary for normal sperm function.[6,7]

It is difficult to establish exactly which microorganisms are involved in the pathogenesis of infertility, but it is extremely important to determine both microbial factors and host factors that contribute to its development, since the commensal microbiota plays a role in the regulation of local immune homeostasis of the biotope.[8,9]

Cytokines are hormone-like peptides that regulate intercellular interactions of all parts of the immune system.[10,11] Currently, secretory inhibitors of cytokines are being actively studied, that is, the ability of cell-free supernatants of microorganisms to reduce the level of cytokines after their cocultivation.[12,13] The important and paramount role of the immune status at the local level in the pathogenesis of chronic prostatitis has also been noted.[14] However, the available research on the immunomodulatory effect of symbiotic bacteria does not allow the effect of microorganisms on the level of cytokines in semen to be assessed. In addition, the study of biofilm formation by microorganisms inhabiting the urethra is of great importance, since this makes it possible for microorganisms to avoid various host antimicrobial immunity proteins, which contribute to the development of infection.[15,16]

This work aimed to determine the level of a secretory inhibitor of cytokines and biofilm formation by the seminal microbiota of healthy and infertile subjects.

PARTICIPANTS AND METHODS

Participants

The study was carried out at the infertility clinic at Orenburg Regional Clinical Hospital No. 1 (Orenburg, Russia). Semen samples from 72 men were collected during the study period from August 2018 to July 2019.

Our research was performed by strict application of criteria following the 2010 World Health Organization (WHO) guidelines.[17] Sperm parameters were determined manually. To exclude errors in the event of abnormal semen quality at the initial examination, a second examination was carried out after 2 weeks. The total volume of the ejaculate, the color, pH, time to liquefaction; total number of spermatozoa per ml of ejaculate, their motility, morphology, agglutination, and aggregation and the presence of leukocytes,

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erythrocytes, and spermatogenic cells were determined. Determination of round cells was the first stage of the study. Subsequently, we differentiated round cells into leukocytes or spermatogenic cells.

Seventy-two subjects were examined. From the data obtained and taking into account anamnesis, the subjects were divided into two groups. Conditionally healthy subjects (n = 30), and infertile subjects (n = 42), in whom changes in the spermiogram of at least two parameters were present and whose partner did not become pregnant after unprotected intercourse for 1 year.

Isolation of strains and determination of microorganism species

A total of 118 strains of microorganisms were isolated from semen using bacteriological methods. Within three hours of collection (in accordance with the WHO recommendations),[17] semen samples (0.1 ml) were inoculated in Endo Agar (BioMedia, Obolensk, Russia), Bi.G.G.Y. Agar (Nickerson's medium; HiMedia Laboratories Pvt., Ltd., Dindori, Nashik, India), and Schaedler Agar (HiMedia Laboratories Pvt., Ltd.) and cultured at 37°C. Obligate anaerobic bacteria were cultured in a CO_2 incubator (Binder, Tuttlingen, Germany) with O_2 from 0.2% to 0.6% (*v/v*) and CO_2 from 5% to 9% (*v/v*). The number of microorganisms in 1 ml of semen was used to determine the colony-forming units (cfu ml^{-1}). Organisms isolated at a concentration of >1 × 10^3 cfu ml^{-1} were considered significant.

To reliably determine the species of microorganisms, isolated strains were identified by 16 small bacterial ribosomal RNA (sRNAs). Total DNA was extracted by Bel'kova.[18] The samples were supplemented with 400 μl of Tris buffer and homogenized using a TissueLyser LT homogenizer (Genecraft Labs, Meruya Utara, Jakarta Barat) with the E lysing matrix (MP Biomedikals, Solon, OH, USA) for 1 min at 50 Hz. The mixture was then supplemented with 50 μl Tris buffer with lysozyme (50 mg ml^{-1}) and incubated for 60 min at 37°C. Sodium dodecyl sulfate at a final concentration of 1% and proteinase K were added. The mixture was incubated for 60 min at 60°C. After extraction with phenol-chloroform-isoamyl alcohol (25:24:1 [*v/v/v*]) and subsequent extraction with a chloroform-isoamyl alcohol mixture (24:1 [*v/v*]), DNA from the aqueous phase was precipitated with absolute ethanol with 5 mol l^{-1} ammonium acetate (1:10 [*v/v*]) at −20°C. DNA was dried and dissolved in Ultra-Pure distilled water (Invitrogen, Loughborough, UK). The DNA concentration was quantified in a Qubit 4.0 Fluorometer with a dsDNA High-Sensitivity Assay Kit (Life Technologies, Carlsbad, CA, USA).

DNA libraries were prepared according to the Quick-16S NGS Library Prep Kit protocol (Zymo Research, Irvine, CA, USA). The library was sequenced in a 2 × 300-nucleotide run using the MiSeq reagent kit version 3 and MiSeq desktop sequencer (Illumina, San Diego, CA, USA). Data analysis was performed on the Local Run Manager platform (Illumina) using 16S Metagenomics Analysis Module version 1.0. The quality of the reads was examined by FastQC version 0.11.7. software (Slashdot Media, San Diego, CA, USA). Paired reads were pooled using PEAR version 0.9.10 software[19] and re-evaluated with FastQC version 0.11.7. The Usearch version 10.0.240 software package was used for filtering and further processing of the pooled reads.[20] Data were filtered with the following parameters: minimum length (fastq_minlen) – 420 base pairs (bp); expected error (fastq_maxee) – no more than 1.0.[21] Chimeric sequences were removed using the UCHIME2 algorithm.[22] The taxonomy of operational taxonomic units (OTUs) was determined with the NCBI Nucleotide BLAST tool using the RefSeq Targeted Loci database (https://blast.ncbi.nlm.nih.gov).

The method was performed using recombinant cytokines and cell-free supernatants of the studied microorganisms.[12]

Cell-free supernatants of microorganisms were obtained from broth culture. Strains were cultivated in Schaedler Broth (HiMedia) at 37°C for 24–48 h, centrifuged for 15 min at 3200*g*, and filter sterilized (0.22-μm pore diameter; MF-Millipore, Merck KGaA, Darmstadt, Germany).

The resulting supernatant was added to recombinant cytokines: interferon-γ (IFN-γ: I3265-1MG), tumor necrosis factor-α (TNF-α: T6674-10UG), interleukin-17 (IL-17: SRP3080), interleukin-1Ra (IL-1Ra: SRP3084), and interleukin-10 (IL-10: I9276-5UG) (Sigma-Aldrich, St. Louis, MO, USA) at a ratio of 1:1 with the experimental samples as follows: IL-10 (56.1 pg ml^{-1}), IL-1 (316.2 pg ml^{-1}), TNF-α (12.1 pg ml^{-1}), IFN-γ (71.9 pg ml^{-1}), and IL-17 (210.0 pg ml^{-1}). A cytokine solution introduced into Schaedler Broth was used as a control. The cell-free supernatant of microorganisms was incubated for 2 h (37°C), and the reaction was stopped by cold exposure. The concentration of cytokines was determined using the immunoenzyme test system (Cytokin, St. Petersburg, Russia), and the results were recorded on an Elx 808 photometer (BioTek, Winooski, VT, USA) at 492 nm. The secretory inhibitor of cytokines is expressed in pg of inactivated cytokines per ml of supernatant in the experiment compared with that of the control (in pg ml^{-1}). The secretory inhibitor of cytokines (in pg ml^{-1}) equals the concentration of cytokines in the control assay (in pg ml^{-1}) minus the concentration of cytokines in the experimental assay (in pg ml^{-1}). The results obtained were compared with a control group of healthy men.

Biofilm assay

Biofilm formation was studied by the adhesion of microorganisms to the surface of a 96-well polystyrene sterile plate.[23,24] The bacterial strains were cultured in Schaedler Agar at 37°C for 24–48 h. A bacterial suspension (10^9 cfu ml^{-1}) was prepared, and 150 μl was added to a 96-well plate. As a control, 150 μl of Schaedler Broth alone was added. After incubation, plankton cells (nonadherent bacterial cells) were removed by shaking the plate and were washed with phosphate-buffered saline (PBS) with crystal violet (CV; concentration of 0.1%). After removal of PBS, the remaining biofilm-bound CV was dissolved in 1 ml of absolute ethanol. The biofilm biomass was quantified by measuring the optical density at 630 nm (OD_{630}) in an Elx 808 (BioTek). The control was the optical density of the Schaedler broth (0.054 OD_{630}). The results are indicated in units of optical density, OD_{630}. All biofilm analyses were performed in three independent experiments. The results obtained were compared with a control group of healthy men.

Statistical analyses

For statistical analyses, Statistica 10.0 (StatSoft, TIBCO, Tulsa, OK, USA) and Excel software programs (Microsoft Office Excel 2010, Redmond, WA, USA) were used. The differences between the groups were calculated with the Student's t-test. Statistical significance was assumed at $P < 0.05$. The prevalence of species was assessed by the relative number in % (M ± m), where M is the relative amount expressed as a percentage, and m is the error of the relative value. All data are presented as mean ± standard deviation (mean ± s.d.).

RESULTS

In 42 subjects (infertile subjects) with chronic bacterial prostatitis, spermogram abnormalities were revealed, mainly in two indicators: mobility of spermatozoa category A + B (mean ± s.d.: 28.4% ± 12.7%) and Kruger normal morphology (mean ± s.d.: 3.8% ± 0.9%), as shown in **Table 1**. The mean ± s.d. of age was 32.5 ± 6.0 years. There was no difference in age between the groups.

Differences in semen volume, sperm concentration, and leukocyte concentration were not significant between the groups.

The species of microorganisms isolated from the semen of healthy and infertile subjects were different. A total of 118 strains of microorganisms were identified from 72 semen samples, which were divided as follows.

The most abundant bacteria in the healthy subjects was *Staphylococcus* (**Table 2**), and the least common types were *Corynebacterium*, *Neisseria*, *Veillonella*, and *Enterococcus*. The most abundant bacteria in the infertile subjects was also *Staphylococcus*. The least common bacteria in this group of subjects were *Corynebacterium*, *Streptococcus*, *Escherichia*, *Actinomyces*, *Bacillus*, *Klebsiella*, *Pseudomonas*, *Georgenia*, and *Propionibacterium* (all $P < 0.05$).

All cell-free supernatants of microorganisms from infertile subjects reduced the level of IL-1, but only some strains reduced the levels of IL-10, TNF-α, IFN-γ, and IL-17 (**Table 3**). Cell-free supernatants of microorganisms from healthy subjects also decreased the levels of IL-10, IL-1, and IL-17.

Cell-free supernatants of *Staphylococcus* from healthy subjects were more able to reduce IL-10 and IL-17 than those from infertile men (**Table 3** and **4**). In particular, *Corynebacterium* from healthy subjects reduced IL-10, IL-1 IFN-γ, TNF-α, and IL-17. Cell-free supernatants of *Enterococcus* of infertile men reduced IL-1 levels and *Corynebacterium* reduced TNF-α levels to a greater extent than those from healthy subjects.

We also evaluated the ability of microorganisms isolated from the semen of conditionally healthy individuals and men with infertility to form biofilms (**Table 5**). *Staphylococcus* and *Enterococcus* species increased the level of biofilm formation in comparison with healthy people. In contrast, *Corynebacterium* reduced biofilm levels in infertile subjects compared to healthy subjects.

Table 1: Comparison of seminal characteristics of ejaculates between healthy subjects and infertile subjects

Parameter	*Healthy subjects (n=30)*	*Infertile subjects (n=42)*
Semen volume (ml)	3.2±1.3	3.4±1.4
Sperm concentration ($\times 10^6$ ml^{-1})	41.4±10.8	38.2±18.6
Sperm motility category A + B (%)	64.1±19.1	28.4±12.7*
Kruger morphology (%)	8.1±2.4	3.8±0.9*
Leukocyte concentration ($\times 10^6$ ml^{-1})	1.8±1.0	2.1±0.6
Spermatogenic cells (%)	2.4±1.2	3.6±1.3

*Significant difference in seminal characteristics of ejaculates in infertile subjects when compared to healthy subjects (mean±s.d.). s.d.: standard deviation

DISCUSSION

Since semen contains various microorganisms, it can serve as a medium for the development of certain diseases.[25,26] The presence of bacteria in seminal fluid may represent a potentially curable cause of male infertility.[27] It has been found that a statistically significant negative effect on the reproductive potential of spermatozoa is caused by *Escherichia coli*, *Ureaplasma urealyticum,* and *Staphylococcus aureus*.[28] Therefore, it is important to understand the species composition of semen bacteria to better understand the etiology and pathogenesis of infections and their relationship with infertility.

In our study of the seminal bacterial communities of infertile and healthy subjects, it was shown that the species of microbiota were more extensive in most semen samples from infertile men than those from healthy semen donors. In addition, the species structure in infertile patients consisted of *Streptococcus*, *Escherichia*, *Actinomyces, Bacillus*, *Pseudomonas*, *Georgenia,* and *Propionibacterium.* Our data showed that *Staphylococcus* was more common in the seminal fluid of infertile men, confirming the data of Lbadin and Ibeh,[29] who identified an association of *Staphylococcus aureus*, *Staphylococcus saprophyticus,* and other pathogens with bacteriospermia and poor general sperm motility and morphology.[30] When studying bacteriospermia, other authors have also often isolated *Staphylococcus aureus* and *Staphylococcus epidermidis*.[5,31,32]

Other authors, when detecting 16s ribosomal RNA genes, identified four of the more common bacterial species: *Enterococcus faecalis*, *Escherichia coli*, and *Staphylococcus aureus*.[33] Results combining next-generation sequencing technology and the clinical study of sperm quality have shown that the most numerous bacteria are *Lactobacillus*, *Pseudomonas*, *Prevotella*, *Gardnerella*, *Rhodanobacter*, *Streptococcus*, *Finegoldia*, and *Haemophilus*. The most common genus in healthy subjects in that study was *Corynebacterium*, which is to some extent consistent with our data. In particular, in infertile patients of our study, three main groups of bacteria were found: *Staphylococcus*, *Corynebacterium,* and *Enterococcus*. We noted some differences from other authors owing to a combination of two methods: classical bacteriological methods (isolation of a pure culture of microorganisms from semen) and identification of microorganisms based on their 16S ribosomal RNAs.

In addition, various properties of the semen microbiota have been actively studied. It has been shown that semen probably has a highly

Table 2: Species of microorganisms isolated from semen of healthy subjects and infertile subjects

Stains (healthy subjects/ infertile subjects)	*Species composition (%), mean±s.d.*		*Microorganism (Log cfu per ml semen), mean±s.d.*	
	Healthy subjects (n=30)	*Infertile subjects (n=42)*	*Healthy subjects (n=30)*	*Infertile subjects (n=42)*
Staphylococcus (18/40)	41.8±1.2	53.3±1.8	3.6±0.1	5.6±0.1
Corynebacterium (6/11)	14.0±1.0	15.0±1.3	5.0±1.0	3.0±0.15
Enterococcus (6/10)	14.0±1.3	13.3±0.9	3.0±0.2	5.6±0.16
Neisseria (8/0)	18.6±0.2	Not detected	8.0±0.9	Not detected
Veillonella (5/0)	11.6±0.8	Not detected	5.0±0.2	Not detected
Streptococcus (0/3)	Not detected	4.0±0.1	Not detected	3.0±0.33
Escherichia (0/3)	Not detected	4.0±0.1	Not detected	5.0±0.5
Actinomyces (0/1)	Not detected	1.3±0.1	Not detected	3.0±0.0
Bacillus (0/1)	Not detected	1.3±0.3	Not detected	3.0±0.0
Klebsiella (0/2)	Not detected	2.6±0.2	Not detected	3.5±0.5
Pseudomonas (0/2)	Not detected	2.6±0.2	Not detected	3.5±0.5
Georgenia (0/1)	Not detected	1.3±0.1	Not detected	3.0±0.0
Propionibacterium (0/1)	Not detected	1.3±0.1	Not detected	3.0±0.0

s.d.: standard deviation; cfu: colony-forming unit

Table 3: Concentration of secretory inhibitors of cytokines in semen from infertile subjects

Cell-free supernatant of strains	*IL-10 (pg ml^{-1})*	*IL-1 (pg ml^{-1})*	*TNF-α (pg ml^{-1})*	*IFN-γ (pg ml^{-1})*	*IL-17 (pg ml^{-1})*
Staphylococcus (*n*=40)	29.1±4.8*	243.5±36.4	10.37±2.8	77.8±11.0	170.8±10.3
Corynebacterium (*n*=11)	188.6±32.9*	247.9±29.1	30.43±4.3*	445.1±51.3*	351.8±20.5*
Enterococcus (*n*=10)	30.5±9.2	219.0±40.8	9.81±2.0	72.7±6.1	112.7±3.4
Streptococcus (*n*=3)	20.9±3.4	293.8±41.2	9.67±0.28	72.4±1.6	143.5±2.2
Escherichia (*n*=3)	26.2±2.2	234.2±20.6	9.51±0.3	77.6±2.4	168.0±6.9
Actinomyces (*n*=1)	30.8±0.0	238.4±0.0	10.2±0.0	92.9±0.0	160.7±0.0
Bacillus (*n*=1)	56.1±0.0	115.2±0.0	9.53±0.0	44.8±0.0	65.8±0.0
Klebsiella (*n*=2)	43.9±5.02	164.9±4.4	12.23±0.4	70.7±1.2	126.7±1.0
Pseudomonas (*n*=2)	44.9±2.8	96.5±4.3	13.1±1.6	44.4±2.3	99.6±2.6
Georgenia (*n*=1)	25.6±0.0	258.3±0.0	9.87±0.0	241.9±0.0	178.2±0.0
Propionibacterium (*n*=1)	21.8±0.0	168.9±0.0	14.32±0.0	72.6±0.0	153.5±0.0

*Significant difference in the concentration of secretory inhibitors of cytokines in infertile subjects compared to healthy controls (mean±s.d.). Control level of cytokines: IL-10 (56.1 pg ml^{-1}), IL-1 (316.2 pg ml^{-1}), TNF-α (12.1 pg ml^{-1}), IFN-γ (71.9 pg ml^{-1}), and IL-17 (210.0 pg ml^{-1}). IL-10: interleukin-10; IL-1: interleukin-1; TNF-α: tumor necrosis factor-α; IFN-γ: interferon-γ; IL-17: interleukin-17; s.d.: standard deviation

Table 4: Concentration of secretory inhibitors of cytokines in semen from healthy subjects

Cell-free supernatant of strains	*IL-10 (pg ml^{-1})*	*IL-1 (pg ml^{-1})*	*TNF-α (pg ml^{-1})*	*IFN-γ (pg ml^{-1})*	*IL-17 (pg ml^{-1})*
Staphylococcus (*n*=18)	18.3±2.6	255.8±21.6	10.5±2.6	73.3±18.6	18.3±4.6
Corynebacterium (*n*=6)	35.7±4.9	239.0±19.5	11.7±1.5	68.2±10.5	35.7±11.8
Enterococcus (*n*=6)	26.9±3.8	255.1±17.1	9.2±2.0	72.5±3.7	26.9±4.2
Neisseria (*n*=8)	17.2±4.2	240.8±20.9	12.2±3.3	68.7±9.3	17.2±9.2
Veillonella (*n*=5)	107.6±30.8	255.8±18.1	21.8±4.1	93.6±17.2	107.6±30.1

*Significant difference in the concentration of secretory inhibitors of cytokines in infertile subjects compared to healthy controls (mean±s.d.). Control level of cytokines: IL-10 (56.1 pg ml^{-1}), IL-1 (316.2 pg ml^{-1}), TNF-α (12.1 pg ml^{-1}), IFN-γ (71.9 pg ml^{-1}), and IL-17 (210.0 pg ml^{-1}). IL-10: interleukin-10; IL-1: interleukin-1; TNF-α: tumor necrosis factor-α; IFN-γ: interferon-γ; IL-17: interleukin-17; s.d.: standard deviation

Table 5: The biofilm-forming capacity of isolated bacterial strains from semen of healthy and infertile men

Strains (healthy subjects/infertile subjects)	*Biofilms produced by microorganisms isolated from healthy subjects (OD_{630})*	*Biofilms produced by microorganisms isolated from infertile subjects (OD_{630})*
Staphylococcus (18/40)	0.46±0.18	0.94±0.14*
Corynebacterium (6/11)	0.45±0.12	0.21±0.09*
Enterococcus (6/10)	0.77±0.12	1.05±0.21*
Neisseria (8/0)	0.46±0.10	Not detected
Veillonella (5/0)	0.30±0.16	Not detected
Streptococcus (0/3)	Not detected	0.82±0.21
Escherichia (0/3)	Not detected	0.80±0.13
Actinomyces (0/1)	Not detected	0.14±0.00
Bacillus (0/1)	Not detected	0.82±0.00
Klebsiella (0/2)	Not detected	0.90±0.06
Pseudomonas (0/2)	Not detected	1.29±0.20
Georgenia (0/1)	Not detected	0.14±0.00
Propionibacterium (0/1)	Not detected	0.87±0.00

*Significant difference in bacterial biofilms between healthy and infertile subjects (mean±s.d.). OD_{630}: optical density at 630 nm; s.d.: standard deviation

effective defense system involving the production of microbiocidal agents, likely comprising peptides or proteins.[8,34]

The secretion of the secretory inhibitor of platelet microbicidal protein (SIPMP) by urethral isolates has also been shown to be correlated with the diagnosis of prostatitis. Moreover, the ability of bacteria to inactivate lysozyme is associated with the development of infertility.[35–37]

Staphylococcus, *Corynebacterium*, and *Enterococcus* isolated from infertile subjects have higher levels of biofilm formation than the same bacteria isolated from healthy men. Other equally important studies related to the study of biofilms of microorganisms in semen have shown that for the fixation of a biotope, bacteria must attach to surfaces of the host.[38,39] Thus, it is necessary to determine the level of biofilm-producing strains isolated from the seminal fluid of infertile subjects.

The presence of various microorganisms in the seminal plasma of healthy and fertile males should be considered from the perspective of their interaction with local antimicrobial defense factors of the host.

Inflammation as a response to an incoming foreign agent involves a complex interplay of signaling molecules known as cytokines and their receptors expressed by host tissues and immune cells. Cytokine networks are the main controlling elements in inflammation and immune reactions that occur in infections. Consequently, these cytokine mediators are often targets of invading microorganisms, disrupting the immune response.

Complex interactions between proinflammatory and anti-inflammatory mediators allow the proper balance to be maintained and prevent the development of pathological, tissue-destructive processes.[40] Therefore, the interaction between bacteria and cytokine networks is an important theme in microbial pathogenicity. Disturbance of this communication network has the potential to profoundly affect the pathological outcome of infectious diseases. One mode of cytokine signaling disruption can occur through proteolytic modification of these molecules and their receptors on cell surfaces.

It has been reported that lysine-specific gingipain *Porphyromonas gingivalis* in the coculture system degrades and inactivates TNF-α, IL-1β, IL-1 receptor antagonist, IL-6, IL-8, IFN-γ, and IL-17A.[41,42]

Group A *Streptococcus* (GAS) protease SpyCEP cleaves IL-8. By inactivating the multifunctional host defense peptide IL-8, the SpyCEP protease impairs neutrophil clearance mechanisms, contributing to the pathogenesis of invasive streptococcal infection.[43]

Pseudomonas aeruginosa secretes several proteases that are considered to be important virulence factors. Two key proinflammatory cytokines, IL-6 and IL-8, are substrates for pseudolysin (elastase) and aeruginolysin (alkaline protease).[44] This phenomenon could help *Pseudomonas aeruginosa* establish an infection, since it blocks the

recruitment of leukocytes.[45] *Pseudomonas aeruginosa* isolated from cystic fibrosis subjects secretes the protease AprA in a LasR-dependent manner, which is able to directly degrade epithelial-derived IFNλ, inhibiting IFN signaling.[46]

Cytokines are physiological seminal components, and their abnormal levels, reported in different pathological conditions, disrupt semen function.[47] Many so-called proinflammatory and immunoregulatory cytokines are produced by testicular somatic and spermatogenic cells, both under normal conditions and in response to inflammatory stimuli.[48] Seminal cytokines are also secreted by immunocompetent cells in various pathogenic conditions, including leukocytospermia.[49]

In our study, microorganisms, such as *Staphylococcus* and *Corynebacterium,* were able to inactivate IL-10. The genus *Corynebacterium* in infertile men, in comparison with conventionally healthy people, also reduced the level of the secretory inhibitor of cytokines in relation to TNF-α and the *Enterococcus* reduced the level of the secretory inhibitor of cytokines IL-1.

This study raises further questions. Can an increase in microbial cytokines affect the local cytokine balance? Thus, further research is required to determine whether a causal relationship exists between microorganism cytokines (IL-10, IL-1, TNF-α, IFN-γ, and IL-17) and male infertility. The answer to this question lies in continuing research in clinical trials.

AUTHOR CONTRIBUTIONS

OVB conceived of the study and participated in its design and coordination. NBP participated in the project coordination, generalization of experimental data, and literature search. EVI identified the isolated microorganisms by genetic methods and bioinformatics processing and helped draft the manuscript. INC studied the secretory inhibitor of cytokines of microorganisms isolated from semen. AVB performed the bacteriological isolation of microorganisms from semen. TAB isolated microorganisms to form biofilms. MDK collected biomaterials and coordinated the clinical trials. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declared no competing interests.

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REFERENCES

1 Deka PK, Sarma S. Psychological aspects of infertility. *BJMP* 2010; 3: 336.
2 Diemer T, Huwe P, Ludwig M, Hauck EW, Weidner W. Urogenital infection and sperm motility. *J Androl* 2003; 35: 283–7.
3 Ochsendorf FR. Sexually transmitted infections: impact on male fertility. *J Androl* 2008; 40: 72–5.
4 Cottell E, Harrison RF, McCaffrey M, Walsh T, Mallon E, *et al*. Are seminal fluid microorganisms of significance or merely contaminants? *Fertil Steril* 2000; 74: 465–70.
5 Rodin DM, Larone D, Goldstein M. Relationship between semen cultures, leukospermia, and semen analysis in men undergoing fertility evaluation. *Fertil Steril* 2003; 79: 1555–8.
6 Weng SL, Chiu CM, Lin FM, Huang WC, Liang C, *et al*. Bacterial communities in semen from men of infertile couples: metagenomic sequencing reveals relationships of seminal microbiota to semen quality. *PLoS One* 2014; 9: e110152.
7 Mändar R, Punab M, Borovkova N, Lapp E, Kiiker R, *et al*. Complementary seminovaginal microbiome in couples. *Res Microbiol* 2015; 166: 440–7.
8 Bukharin OV, Kuzmin MD, Ivanov YB. [The role of the microbial factor in the pathogenesis of male infertility]. *J Microbiol Epidemiol Immunobiol* 2000; 2: 106–10. [Article in Russian].
9 Bukharin OV, Chainikova IN, Ivanova EV, Perunova NB, Bondarenko TA, *et al*. [Immunoregulatory profile of microsymbionts of the human intestinal biotope]. *J Microbiol Epidemiol Immunobiol* 2018; 4: 42–51. [Article in Russian].
10 Yarilin AA. [Fundamentals of Immunology]. Moscow: Medicine; 1999. p608. [Book in Russian].
11 Diemer T, Hales DB, Weidner W. Immune-endocrine interactions and Leydig cell function: the role of cytokines. *J Androl* 2003; 35: 55–63.
12 Bukharin OV, Perunova NB, Chainikova IN, Ivanova EV, Smolyagin AI, *et al*. [Anti-cytokine activity of microorganisms]. *J Microbiol Epidemiol Immunobiol* 2011; 4: 56–61. [Article in Russian].
13 Bondarenko TA, Danilova EI, Chainikova IN, Ivanova EV, Chelpachenko OE, *et al*. [Antipeptide activity of intestinal microsymbionts for assessing the state of intestinal homeostasis]. *Russ J Immunol* 2018; 3: 222–6. [Article in Russian].
14 Konoplya AI, Shatokhin MN, Gavriliuk VP. [Immunologic problems of the chronic prostatitis]. *Immunopatol Allergol Infectol* 2015; 2: 29–34. [Article in Russian].
15 Greiner LL, Edwards JL, Shao J, Rabinak C, Entz D, *et al*. Biofilm formation by *Neisseria gonorrhoeae*. *Infect Immun* 2005; 73: 1964–70.
16 Mazzoli S. Biofilms in chronic bacterial prostatitis (NIH-II) and in prostatic calcifications. *FEMS Immunol Med Microbiol* 2010; 59: 337–44.
17 World Health Organization. WHO Laboratory Manual for the Examination and Processing of Human Semen. 5th edition. Geneva: World Health Organization; 2010.
18 Bel'kova NL, Andrreeva AM. [Introduction to Molecular Ecology of Microorganisms. Study Guide]. Yaroslavl: OOO Printhouse; 2009. p56–63. [Article in Russian].
19 Zhang J, Kobert K, Flouri T, Stamatakis A. PEAR: a fast and accurate Illumina Paired-End reAd merger. *Bioinformatics* 2014; 30: 614–20.
20 Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods* 2013; 10: 996–8.
21 Edgar RC, Flyvbjerg H. Error filtering, pair assembly and error correction for next-generation sequencing reads. *Bioinformatics* 2015; 31: 3476–82.
22 Edgar RC. UCHIME2: improved chimera prediction for amplicon sequencing. Available from: https://www.researchgate.net/publication/345814794_UCHIME2_improved_chimera_prediction_for_amplicon_sequencing. [Last accessed on 2021 Sep 24].
23 Merritt JH, Kadouri DE, O'Toole GA. Growing and analyzing static biofilms. *Curr Protoc Microbiol* 2005; 7: Unit 1B.1.
24 O'Toole GA, Kolter R. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol* 1998; 28: 449–61.
25 Gallo MF, Warner L, King CC, Sobel JD. Association between semen exposure and incident bacterial vaginosis. *Infect Dis Obstet Gynecol* 2011; 2011: 842652.
26 Ott MA, Katschke A, Tu W, Fortenberry JD. Longitudinal associations among relationship factors, partner change, and sexually transmitted infection acquisition in adolescent women. *Sex Transm Dis* 2011; 38: 153–7.
27 Pellati D, Mylonakis I, Bertoloni G, Fiore C, Andrisani A, *et al*. Genital tract infections and infertility. *Eur J Obstet Gynecol Reprod Biol* 2008; 140: 3–11.
28 Sanocka-Maciejewska D, Ciupinska M, Kurpisz M. Bacterial infection and semen quality. *J Reprod Immunol* 2005; 67: 51–6.
29 Lbadin OK, Ibeh IN. Bacteriospermia and sperm quality in infertile male patient at University of Benin Teaching Hospital, Benin City Nigeria. *Mal J Microbiol* 2008; 4: 65–7.
30 Owolabi AT, Fasubaa OB, Ogunniyi SO. Semen quality of male partners of infertile couples in Ile-Ife, Nigeria. *Niger J Clin Pract* 2013; 16: 37–40.
31 Alekwe L, Osamudiamen AI, Aberare LO. Association between bacteriospermia and abnormal semen characteristics. *Pak J Med Health Sci* 2013; 7: 3–6.
32 Domes T, Lo KC, Grober ED, Mullen JB, Mazzulliet T, *et al*. The incidence and effect of bacteriospermia and elevated seminal leukocytes on semen parameters. *Fertil Steril* 2012; 97: 1050–5.
33 Fair WR, Parrish RF. Antibacterial substances in prostatic fluid. *Prog Clin Biol Res* 1981; 75: 247–64.
34 Hall SH, Hamil KG, French FS. Host defense proteins of the male reproductive tract. *J Androl* 2002; 23: 585–97.
35 Ivanov YB, Gritsenko VA, Kuzmin MD. Phenotypic differences between coryneform bacteria isolated from seminal fluid of healthy men and men with chronic prostatitis syndrome. *Asian J Androl* 2009; 11: 517–20.
36 Ivanov YB, Gritsenko VA, Kuzmin MD. Distribution of secretory inhibitor of platelet microbicidal protein among urethral isolates with its correlation with prostatitis. *Asian J Androl* 2008; 10: 189–92.
37 Kuzmin MD, Ivanov YB, Mikhailova EA, Bukharin OV. [The role of persisting microflora in the development of pathospermia]. *Urol Nefrol (Mosk)* 1998; 2: 46–8. [Article in Russian].
38 Anderson MT, Byerly L, Apicella MA, Seifert HS. Seminal plasma promotes *Neisseria gonorrhoeae* aggregation and biofilm formation. *J Bacteriol* 2016; 198: 2228–35.
39 Türk S, Mazzoli S, Štšepetova J, Kuznetsova J, Mändar R. Coryneform bacteria in human semen: inter-assay variability in species composition detection and biofilm production ability. *Microb Ecol Health Dis* 2014; 2: 25.
40 Hessle CC, Andresson B, Wold AE. Gram-positive and Gram-negative bacteria elicit different patterns of pro-inflammatory cytokines in human monocytes. *Cytokine* 2005; 30: 311–8.

41 Stathopoulou PG, Benakanakere MR, Galicia JC, Kinane DF. The host cytokine response to *Porphyromonas gingivalis* is modified by gingipains. *Oral Microbiol Immunol* 2009; 24: 11–7.
42 Akiyama T, Miyamoto Y, Yoshimura K, Yamada A, Takami M, *et al*. *Porphyromonas gingivalis* – derived lysine gingipain enhances osteoclast differentiation induced by tumor necrosis factor-α and interleukin-1β but suppresses that by interleukin-17A: importance of proteolytic degradation of osteoprotegerin by lysine gingipain. *J Biol Chem* 2014; 289: 15621–30.
43 Zinkernagel AS, Timmer AM, Pence MA, Locke JB, Buchanan JT, *et al*. The IL-8 protease SpyCEP/ScpC of group A *Streptococcus* promotes resistance to neutrophil killing. *Cell Host Microbe* 2008; 4: 170–8.
44 Matheson NR, Potempa J, Travis J. Interaction of a novel form of *Pseudomonas aeruginosa* alkaline protease (aeruginolysin) with interleukin-6 and interleukin-8. *Biol Chem* 2006; 387: 911–5.
45 LaFayette SL, Houle D, Beaudoin T, Wojewodka G, Radzioch D, *et al*. Cystic fibrosisadapted *Pseudomonas aeruginosa* quorum sensing lasR mutants cause hyperinflammatory responses. *Sci Adv* 2015; 1: e1500199.
46 Sörensen M, Kantorek J, Byrnes L, Boutin S, Mall MA, *et al*. *Pseudomonas aeruginosa* modulates the antiviral response of bronchial epithelial cells. *Front Immunol* 2020; 11: 96.
47 Moretti E, Cerretani D, Noto D, Signorini C, Iacoponi F, *et al*. Relationship between semen IL-6, IL-33 and malondialdehydegeneration in human seminal plasma and spermatozoa. *Reprod Sci* 2021; 28: 2136–43.
48 Loveland KL, Klein B, Pueschl D, Indumathy S, Bergmann M, *et al*. Cytokines in male fertility and reproductive pathologies: immunoregulation and beyond. *Front Endocrinol (Lausanne)* 2017; 8: 307.
49 Fraczek M, Czernikiewicz A, Kurpisz M. Cytokines and oxidative stress in the germ line. In: Agarwal A, Aitken RJ, Alvarez JG, editors. Studies on Men's Health and Fertility. Totowa: Humana Press; 2012. p179–205.