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ORIGINAL PAPER

Molecular analysis of microorganisms in the semen and their impact on semen parameters

Jenniffer Puerta Suárez [1], Juan Carlos Hernandez [2], Walter Dario Cardona Maya [1]

[1] Grupo Reproducción, Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad de Antioquia - UdeA, Medellín, Colombia;
[2] Infettare, Facultad de Medicina, Universidad Cooperativa de Colombia, Medellín, Colombia.

Summary ***Objective:** Chronic genitourinary infections can alter male fertility and even promote carcinogenic processes. This study aimed to evaluate the effect of the presence in the semen of microorganisms on semen quality.*
***Materials and methods:** Clinical symptoms and conventional and functional seminal parameters of eleven fertile donors and ten volunteers with prostatitis-like symptoms were evaluated. Nitric oxide, antioxidant capacity, and pro-inflammatory cytokines in semen and seminal plasma samples were also quantified. Finally, the expression of the ROR-γT, FoxP3, and T-bet genes in semen and the presence of DNA of microorganisms associated with prostatitis in urine and semen were evaluated.*
***Results:** When compared with fertile donors, volunteers with chronic prostatitis-like symptoms reported erectile dysfunction (0% vs. 10%, p = 0.2825) and premature ejaculation (0% vs. 40%; p = 0.0190). No statistically significant differences were observed in seminal parameters, cytokine measurement, antioxidant capacity, nitric oxide concentration and ROR-γT, FoxP3, T-bet. Microorganisms responsible for sexually transmitted infections and some bacteria associated with the microbiota and infections in the prostate gland were detected. In the semen from the subjects with prostatitis-like symptoms T. vaginalis DNA was detected; in addition, N. gonorrhoeae DNA was also detected in semen and urine samples. S. pyogenes was detected in the urine samples from the control group.*
***Conclusions:** Prostatitis-like symptoms are a common finding in young men that affect sexual and reproductive health, but not always the seminal parameters or fertility. The presence of prostatitis-like symptoms does not affect seminal quality. However, it appears to be associated with an increased likelihood of erectile dysfunction and premature ejaculation. Thus, affecting the quality of life and sexual and reproductive health.*

KEY WORDS: *Prostatitis; Fertility; Infection; Inflammation; Seminal quality; Sexual health.*

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INTRODUCTION

Frequent exposure of the prostate to infectious processes can promote chronic inflammation (1), alter fertility (2-4), and even promote cancer (5-7). The role of infection, microbiota and inflammation on male fertility is still controversial (8). *In vitro* studies have shown how microorganisms can affect sperm function by altering motility, inducing apoptosis, increasing reactive oxygen species, altering DNA and the acrosomal reaction, and other factors (8-14). However, these results should be interpreted cautiously because the semen of both fertile and infertile men contains microbiota, mainly bacterial (8).

Urogenital infections in men are less frequent than in women, although they can trigger chronic inflammatory processes such as prostatitis (15). Clinically, prostatitis is classified into four types: i) acute bacterial prostatitis; ii) chronic bacterial prostatitis; iii) chronic pelvic pain syndrome; and iv) asymptomatic inflammatory prostatitis (16-18). Chronic bacterial prostatitis is responsible for 5 to 10% of total prostatitis cases, and at least 30% of those involve recurrent urinary infections (18). It is estimated that 5 to 10% of acute genitourinary infectious and inflammatory processes end in chronic prostatitis (16).

Therefore, this work aimed to evaluate the effect of the presence of microorganisms in the semen on seminal quality and inflammatory markers.

MATERIALS AND METHODS

Study participants

This project was approved by the *Bioethics Committee for research in humans* at the *Institute of Medical Research, Medical School, University of Antioquia* (*Act number 006, April/2018*). Ten subjects with chronic prostatitis-like symptoms and eleven fertile donors asymptomatic for urogenital infections volunteered to participate to the study.

The *National Institute of Health of chronic prostatitis symptoms index* (NIH-CPSI) (19) translated and validated into *Spanish* (20) was employed to select the volunteers according to the criteria reported by *Nickel et al.* (21).

The questionnaire contains 13 items that are scored in three discrete domains: pain, urinary symptoms, and the impact on quality of life.

We considered as fertile donors those who had children under two years or their partner in pregnancy at study recruitment. To be included in the study they should have no history of any genitourinary symptoms, instrumentation, or surgery, and NIH-CPSI total score lesser than 3. On the other hand, the inclusion criteria for the chronic prostatitis-like group were aged > 18 years and presence of prostatitis-like syndrome longer than three months (pain and/or discomfort in the perineum or on ejaculation) with a score in the pain domain of the NIH-

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CPSI greater than 4. After agreeing with their participation in the study, all individuals were required to sign an informed consent. Each volunteer gave a semen sample and a urine mid-stream sample. A blood sample was also taken by qualified personnel in a red Vacutainer tube (*Becton Dickinson, NJ, USA*) to obtain the serum.
Finally, participants also filled out a survey including information on sociodemographic factors, lifestyle, urinary symptoms, and relevant other aspects of sexual and reproductive health that allowed us to identify factors associated with prostatitis symptoms.

Semen collection and analysis
Semen samples were collected into a sterile sample cup through masturbation after sexual abstinence for 2 to 5 days.

Conventional seminal parameters
Volume, progressive motility, concentration, and sperm morphology were evaluated according to parameters established by the *World Health Organization* in the fifth edition of its *Human Semen Processing Manual* (22, 23). The sperm concentration was evaluated using the Makler chamber (22, 23).

Functional seminal parameters
Sperm mitochondrial membrane potential (24), sperm membrane integrity (25), chromatin structure assay (26), sperm membrane lipoperoxidation (27), and intracellular levels of *reactive oxygen species* (ROS) (24) were evaluated by flow cytometry (*Fortessa-Becton Dickinson, NJ, USA*), according to previously established protocols in our lab (24, 28, 29), and analyzing between 5,000 and 10,000 sperm cells. Data were plotted and processed using the FlowJo 7.6 (*Tree Star, Inc. Oregon, USA*).

Seminal plasma total antioxidant capacity evaluation
Three mL of DPPH (2,2-diphenyl-1-picrylhydracil) were mixed with 200μL of the sample. After one hour of incubation, the sample was read in a spectrophotometer (*Spectronic 20 Spectrophotometer®; Genesys, Rochester, NY, USA*) at 515 nm, used ascorbic acid as a positive control (28, 29).

Nitric oxide determination
Nitric oxide quantification was performed using the commercial Griess Reagent Kit for nitrite determination (*Molecular probes, Oregon, USA*) according to the manufacturer's instructions and after deproteinization of the semen and serum samples according to the Serafini method (30) as previously reported (29).

Cytokine quantification
Quantification of IL-12p70, IL-10, IL-1α, IL-6, IL-8, TNF, IL-2, IL-4, IL-17, and IFN-α was performed by BD *Cytometric Bead Array* (CBA) in semen samples (*Human Inflammatory Cytokines Kit, and Human Th1/Th2/Th17 Cytokine Kit, Becton Dickinson, NJ, USA*), and the analysis was carried out in the FlowJo 7.6 as previously reported (29). Forkhead box P3 transcription factor (FoxP3), T-box 2 (T-bet), and retinoid-related orphan receptor γt (RORγ-T) mRNA expression. Total RNA extraction was performed from 200 μL of semen sample using a commercial kit (*Qiagen RNeasy Mini Kit, QIAGEN, Hilden, Germany*). The RNA was used to synthesize cDNA using the commercial RevertAid H Minus First Strand cDNA Synthesis kit (*Thermo Fisher Scientific, Waltham, Massachusetts, USA*). With the cDNA obtained, polymerase chain reactions were performed in real-time for the FoxP3 (Forward: 5-CAGCACATTCCCAGAGTTCCTC-3; Reverse: 5-GCGTGTGAACCAGTGGTAGATC-3); ROR-γt (Forward: 5-TTTTCCGAGGATGAGATTGC-3; Reverse: 5- CTTTCCACATGCTGGCTACA-3), and T-bet (Forward: 5-GCCTACAGAATGCCGAGATTACT-3; Reverse: 5-GGATGC TGGTGTCAACAGATG-3) genes. The gene expression levels were normalized using ΛCt with β-actin (31).

Bacterial detection in semen specimens by PCR assays

DNA extraction
DNA extraction was performed using the phenol-chloroform technique using 500 μL of the semen sample and the 10 mL urine pellet. Briefly, the semen samples were centrifuged at 200g for 10 minutes, and the urine samples were centrifuged at 22000 g for 10 minutes. For each urine or semen sample, 0.5 mL of lysis solution (1M Tris, 0.5M EDTA, 5M NaCl, 10% SDS, and 0.1% triton x-100) and 5 μL of proteinase K were added for 12 hours at 54°C. Subsequently, 1 mL of phenol-chloroform-isoamyl was added, and it was centrifuged at 5000 g for 10 min. Then, 1mL of absolute ethanol (-20°C), 50 μL of 3M sodium acetate was added to the recovered supernatant, and it was left at -20°C overnight to precipitate the DNA.
Finally, it was washed with 1 mL of 70% ethanol; the ethanol was allowed to dry, the DNA was diluted in 100 μL of DNAse/RNAse-free water and quantified in a Nanodrop 2000 Spectrophotometer (*Thermo Scientific, Massachusetts, USA*).

Polymerase chain reaction
The final 25μL reaction volume contained 12.5μL of Master Mix (*Thermo-Scientific, Massachusetts, USA*), a solution containing 0.025 U/L of Taq DNA polymerase, 2 mM of MgCl2, and 0.2 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), 0.2M of each primer, 2 μL of DNA (200 ng), and 9.3 μL of water were added to each reaction. The PCR was carried out in a T3000 thermal cycler (*Whatman, Biometra, Goettingen, Germany*); cycling conditions consisted of an initial denaturation step at 94-95ºC for 5 min, followed by 35-40 cycles of specific conditions as previously, and a final elongation of 5-10 min at 72°C, using primers and following PCR conditions previously described (32) for *β-actin* (33), *Chlamydia trachomatis* (34), *Escherichia coli* (35), *Klebsiella pneumnoniae* (36), *Lactobacillus spp* (37), *Mycoplasma genitalium* (34), *Neisseria gonorrhoeae* (34), *Ochrobatrum atrophy* (38), *Pseudomonas aeruginosa* (36), *Staphylococcus aureus* (36), *Staphylococcus epidermidis* (39), *Streptococcus agalactiae* (40), *Streptococcus pneumoniae* (41), *Streptococcus pyogenes* (42), *Treponema pallidum* (34), *Trichomonas vaginalis* (34), Universal bacteria 27F y 1942R (43), *Ureaplasma urealyticum* (44), *Herpes simplex virus I* (34) and II (34), and *Human papillomavirus* (34). DNA extracted from each bacterial strain or clinical isolates obtained from patients was a positive reaction control. *Lactobacillus spp.* DNA

was obtained from a woman's vaginal smear on day 14 of her menstrual cycle.

Serum prostate-specific antigen (PSA) quantification

According to the manufacturer's instructions, total serum PSA quantification was performed using the commercial total PSA kit (*DiaMetra, Perugia, Italy*). PSA values greater than 4 ng/mL were considered positive, as previously reported (29).

Statistical analysis

A chi-square and a Mann Whitney test were used to compare both groups' dichotomous and numerical variables. The data were analyzed using the statistical program Graph Pad Prism 6.0 (*GraphPad, San Diego, CA, USA*), and a value of $p < 0.05$ was considered significant.

RESULTS

Eleven fertile donors (median age of 32 years) and ten chronic volunteers with prostatitis-like symptoms (median age of 39.5 years) (p = 0.5219) were included in the study (Table 1). Mean body mass index was similar in the two groups (fertile donors 25.7 *vs.* prostatitis-like subjects 23.4, p = 0.2299). Only 40% of the prostatitis-like group were married or living with a partner, compared to 100% of the control group (p = 0.0099). Erectile dysfunction (10%) and premature ejaculation (40%) were self-reported by the subjects with prostatitis-like symptoms (p = 0.2825 and p = 0.0190, respectively). In addition, 50% of subjects with prostatitis-like symptoms reported a history of chronic diseases and stress (p = 0.0072 and p = 0.0382), and 70% reported feeling anxiety (p = 0.0166) associated with their symptoms. Three subjects with prostatitis-like symptoms were excluded from the seminal quality analysis because they reported being vasectomized. No statistically significant differences were found on other sexual health and reproductive aspects evaluated, nor on conventional or functional seminal parameters evaluation.

No differences were found between groups in seminal plasma antioxidant capacity, plasma/serum nitrites concentration, or PSA determinations (Table 2).

The IL-12p70, IL-10, IL-1β, IL-6, IL-8, TNF, IL-2, IL-4, IL-17, IFN-γ cytokines concentrations were evaluated in serum and seminal plasma samples without finding differences (Table 3).

Table 1.
Sociodemographic characteristics.

Characteristics	Control group n = 11	Prostatitis group n = 10	P-value
Children	100	40	0.0034
Education level			0.1005
High school	9.1	0	
Technician	0	10	
University	18.2	60	
Postgraduate	72.7	30	
Marital status			0.0099
Unmarried	0	50	
Married	100	40	
Divorced	0	10	
Number of sexual partners			0.5250
None	0	10	
One to three	36.4	40	
More than three	63.6	50	
Type of sex			
Masturbation	63.6	70	0.7574
Vaginal	100	60	0.0197
Oral	63.6	60	0.8639
Anal			0.2568
Insertive	36.4	40	0.5366
Receptive	0	20	0.1189
Condom use			0.3580
Always	0	10	
Frequently	18.2	20	
Rarely	64.6	30	
Never	18.2	40	

Chi-square. Data indicate percentage.

Table 2.
Seminal parameters, nitric oxide concentration and serum PSA.

Parameters	Control group	Prostatitis group	P-value
Volume (mL)	2.0 (1.5-4.7)	3.5 (1.5-11.8)	0.4556
Progressive motility (%)	49.0 (19.0-81.0)	49.0 (6.0-67.0)	0.7414
Concentration/mL	100.0 (40.5-270.0)	182.0 (7.0-254.0)	0.3269
Viability (%)	79.0 (76.0-91.0)	82.0 (49.0 -85.0)	0.8485
Normal morphology (%)	5.2 (4.2-8.7)	4.6 (4.2-7.0)	0.3874
Teratozoospermia index	1.25 (1.10-1.52)	1.18 (1.12-1.37)	0.4091
High mitochondrial membrane potential (%)	61.3 (33.5-73.5)	66.6 (12.3-75.5)	0.3167
Plasma membrane integrity (%)	63.9 (37.8-84.4)	63.1 (12.1-70.0)	0.8095
ROS production (%)	63.0 (34.8-86.2)	56.9 (17.7-66.8)	0.3612
DNA fragmentation index (%)	10.9 (10.4-14.9)	10.6 (10.3-14.3)	0.5795
Membrane lipoperoxidation (%)	66.8 (9.1-93.3)	71.1 (44.9-96.9)	0.3269
Total antioxidant seminal plasma capacity (%)	61.0 (22.1-81.4)	62.3 (9.5-69.7)	> 0.9999
Seminal plasma nitric oxide concentration (Nitritos μM)	1.25 (0.55-11.7)	0.55 (0.23-2.16)	0.1384
Serum nitric oxide concentration			
(Nitritos μM)	4.6 (1.6-13.0)	2.8 (1.2-7.4)	0.1728
Serum PSA (ng/mL)	0.0 (0.0-18.1)	0.45 (0.0-120.0)	0.3292

Mann Whitney test. Data presented as median and range. ROS: Reactive oxygen species; PSA: Prostatic-specific antigen.

Table 3.
Detection of cytokines in seminal plasma and serum.

	Cytokine pg/mL	Control	Prostatitis-like symptoms	P-value
Seminal plasma	IL-12p70	0 (0-68.9)	8.5 (0-107.5)	0.5907
	IL-1β	0 (0-36.8)	2.7 (0-31.53)	0.7260
	IL-6	8.9 (0-86.9)	7.1 (0-101.9)	0.9159
	IL-8	1808 (0-4202)	1692 (680.4-4334)	> 0.999
	TNF	0 (0-83.8)	24.7 (0-127.5)	0.1553
	IL-2	3.9 (0-23.5)	11.4 (4.3-45.7)	0.2940
	IL-4	0 (0-13.7)	0 (0-19.3)	0.1454
	IL-10	0 (0-26.7)	1.3 (0-19.3)	0.9113
	IL-17	9.3 (0-73.1)	6.6 (0-429.9)	0.8749
	IFN-γ	0 (0-5.9)	0 (0-33.5)	0.6084
Serum	IL-12p70	0 (0-304.3)	0 (0-95.1)	> 0.9999
	IL-1β	0 (0-54.3)	0 (0-2.7)	0.3246
	IL-6	0 (0-15.6)	0 (0-14.9)	0.2479
	IL-8	23.6 (0-301.3)	9.6 (0-66.1)	0.6668
	TNF	0 (0-92.7)	0 (0-30.4)	0.3128
	IL-2	4.9 (2.1-36.0)	8.2 (4.1-47.3)	0.2439
	IL-4	0 (0-19.4)	0 (0-27.0)	0.3128
	IL-10	0 (0-10.5)	0 (0-13.1)	0.5573
	IL-17	0 (0-182.7)	9.9 (0-85.6)	0.2757
	IFN-γ	0.3 (0-12.8)	1.3 (0-6.3)	0.6476

Mann Whitney. Data presented as median and range.

Figure 1.
ROR-γT, FoxP3 and T-bet genes expression.

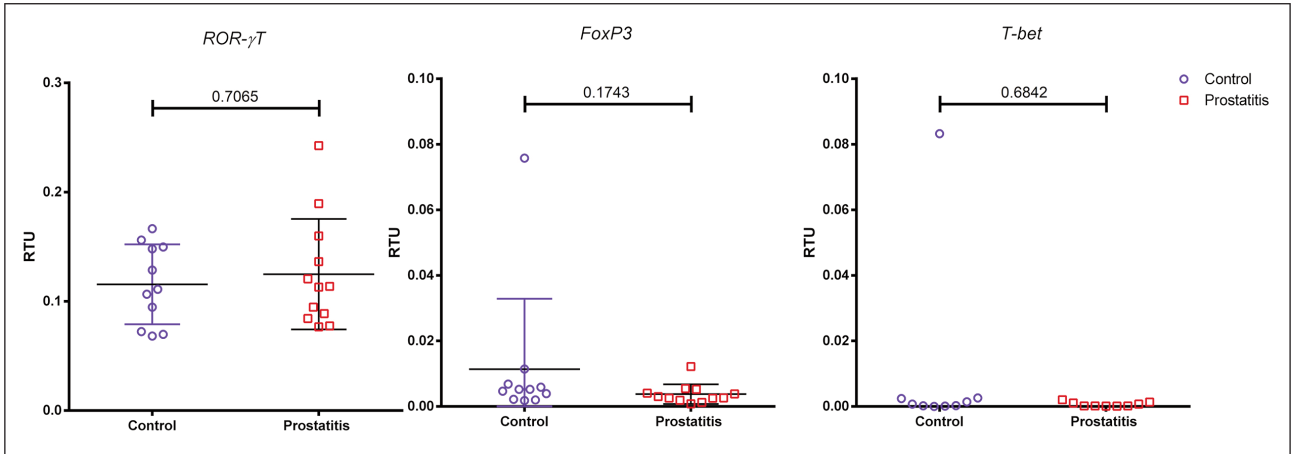


Figure 2.
STIs DNA detection.

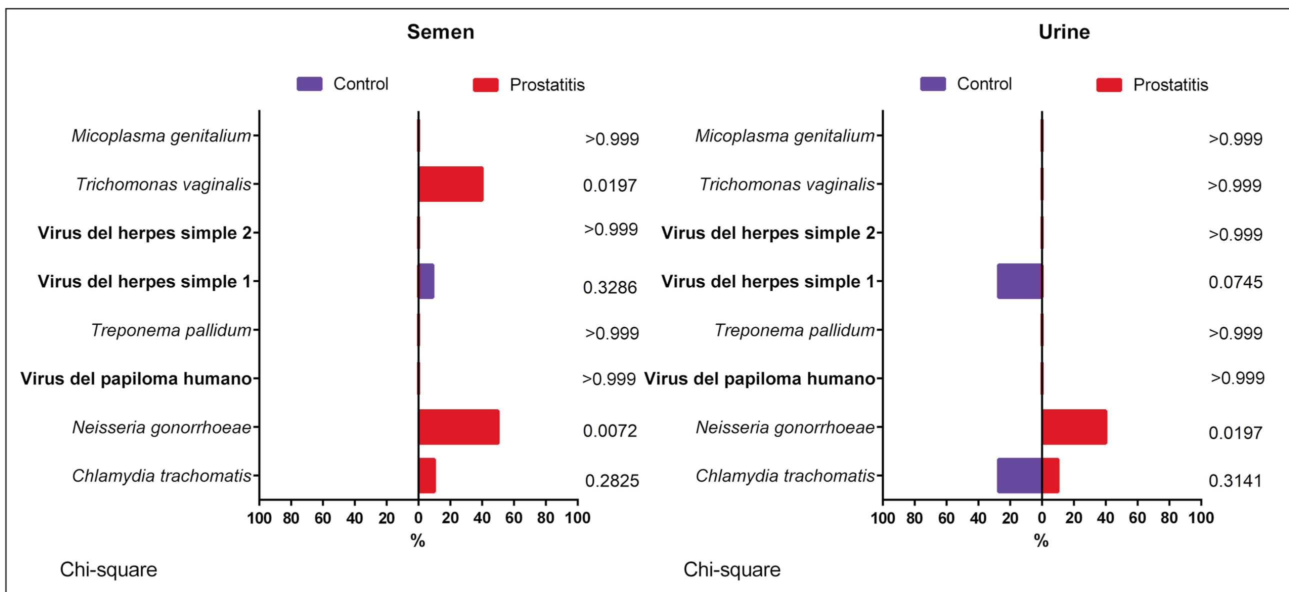
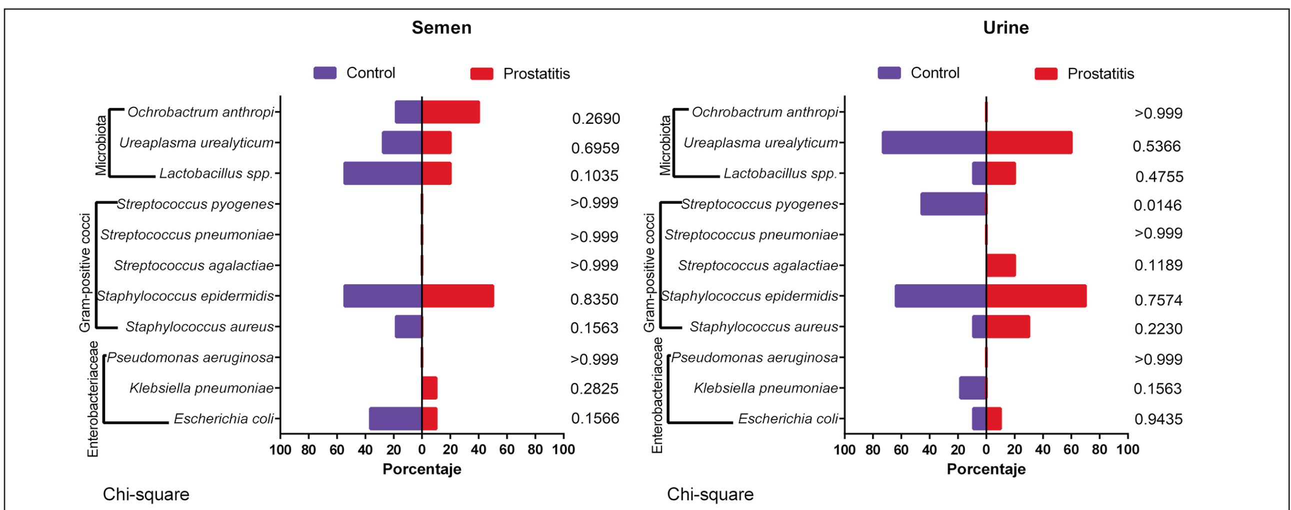


Figure 3.
DNA detection from other bacteria associated.



We also found no statistical difference in the expression of ROR-γT, FoxP3, and T-bet genes in semen samples (Figure 1). Finally, we detected microorganisms responsible for STIs (Figure 2) and some bacteria associated with the microbiota and infections of the prostate gland (Figure 3).
T. vaginalis DNA was detected in 40% of the semen samples of the subjects with prostatitis-like symptoms (p = 0.0197). Furthermore, *N. gonorrhoeae* DNA was detected in in 50% and 40% of semen and urine samples of this group (p = 0.0072 and p = 0.0197). *S. pyogenes* was detected in 45.5% of the urine samples from the control group volunteers (p = 0.0146).

Discussion

Male factor is responsible in 50% of infertility cases, highlighting urogenital infections as the leading causes (8, 45). Urinary tract infections are the most common type of infection in humans, with an estimated annual prevalence of 150 million, representing a high financial impact (46).
In men, urogenital infections are a risk factor for prostatitis development, a disease that dramatically impacts mental and sexual health and quality of life (19, 47). Chronic prostatitis is a common but poorly understood disease that affects men of any age regardless of their geographical origin (15, 21). Prostatitis has been associated with detriments in seminal quality and affects male fertility (4).
In the present study, the seminal quality of fertile donors asymptomatic for urogenital infections was compared with that of men with symptoms of chronic prostatitis without finding significant differences in the conventional or functional parameters. Volunteers with prostatitis-like symptoms had 75 and 82% greater semen volume and concentration than the control group of fertile donors, although the difference did not reach statistical relevance. Similar findings were obtained by *Shang et al.* (18).
N. gonorrhoeae and *T. vaginalis* were detected more frequently in volunteers with prostatitis-like symptoms. Both were also observed in the semen of infertile men being globally prevalent although easily treatable (48).
In addition, the genome of other microorganisms, as *Propionibacterium acnes*, was frequently observed in the semen and urine of patients with recurrent urinary tract infections (49), and the presence of *T. vaginalis* in the urogenital tract was also associated with an increase of the risk of prostate cancer (50).
In fact, there is a close relationship between urogenital infections and prostate cancer. It is estimated that one in five neoplasms could be attributed to microorganisms (1), and prostatitis was considered as a risk factor for cancer development (7, 51).
However, microorganisms are not a synonymous of disease, because it has been described that the microbiota modulates the immune system; for example, *Lactobacillus spp.* is a protective factor in prostatitis (52).
On the other hand, in chronic pelvic pain syndrome animal models, infiltration of macrophages and CD4+ T cells has been observed, which according to the local microenvironment, can differentiate into Th1, Th2, Th17, or Treg (regulatory) cells (7). Chronic prostatitis patients show specific Th1 and Th17 immune responses to prostate antigen associated with chronic inflammation of the male genital tract, which may be the basis for the induction and development of chronic pelvic pain (53). Among the T cell subpopulations are Treg cells that secrete transforming growth factor β (TGF-β) and IL-10 and express the FoxP3 transcription factor. Th17 lymphocytes secrete IL-17 and IL-22 and express the transcription factor ROR-γT with a critical role in infections and tumors. Th1 cells secreting IFN-γ cytokines, IL-2 and TNF-α express the transcription factor T-bet and are essential in developing autoimmune prostatitis (7). Activation of Th1 and Th17 profiles inhibits Treg cells' action, promoting the appearance of chronic pelvic pain (7). Therefore, we assessed in semen samples the mRNA expression of lineage-specifying transcription factors FoxP3, T-bet, and RORγ-T.
Furthermore, although we evaluated several essential aspects of sexual and reproductive health and lifestyle, other variables not evaluated in this study including the impact of ejaculation delayed and intercourse interruptions, were described as risk factors for chronic prostatitis (54). Even urinary retention and anxiety are risk factors for chronic prostatitis (7). However, this is an excellent approach to evaluating prostatitis's effect on male fertility and understanding the relationship between the urogenital microbiota, infection, and inflammation.
The present study is an interesting approach, as a baseline, to understand the impact on the fertility of chronic prostatitis. Although prostatitis does not seem to alter the seminal quality, it seems to impact on fertility by promoting the appearance of other diseases such as erectile dysfunction and premature ejaculation. However, a limitation of the present study is the limited number of subjects included in the study could explain the lack of difference observed in the comparison of microbiology and immune response between controls and subjects with prostatitis-like symptoms.

Conclusions

Although chronic prostatitis is a disease that affects the quality of life, it does not appear to affect seminal parameters. However, chronic prostatitis seems to be related to alterations in sexual function, such as premature ejaculation and erectile dysfunction.

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Correspondence

Jenniffer Puerta Suárez
jenniffer.puerta@udea.edu.co

Grupo Reproducción, Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad de Antioquia, Medellín (Colombia)

Juan Carlos Hernandez
juankhernandez@gmail.com

Infettare, Facultad de Medicina, Universidad Cooperativa de Colombia, Medellín (Colombia)

Walter Dario Cardona Maya (Corresponding Author)
wdario.cardona@udea.edu.co

Grupo Reproducción, Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad de Antioquia, Medellín (Colombia)