ASSOCIATION OF UREAPLASMA UREALYTICUM WITH ABNORMAL REACTIVE OXYGEN SPECIES LEVELS AND ABSENCE OF LEUKOCYTOSPERMIA

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ABSTRACT

Purpose: Ureaplasma urealyticum is a commensal of the lower genitourinary tract of many sexually active adults. The organism is more common in partners of infertile than fertile marriages. We conducted a prospective study at our tertiary care center to confirm a possible association between U. urealyticum and abnormal sperm function parameters.

Materials and Methods: A total of 50 consecutive male patients seeking general urology consultation for lower urinary tract symptoms characteristic of chronic prostatitis were evaluated. Urine and semen localization cultures were performed with additional semen cultures for U. urealyticum, Chlamydia trachomatis and Mycoplasma hominis. Specimens from 21 healthy men were used as controls. Specimens were analyzed by a computer assisted semen analyzer, and verified manually for concentration, percent motility and morphology. Leukocytospermia was measured by the Endtz test. Semen specimens were also analyzed for reactive oxygen species (ROS), acrosome reaction and mannose binding assay.

Results: Of the patients 17 had positive U. urealyticum cultures and the other cultures were negative. Patients with U. urealyticum had significantly higher ROS levels (log \([ROS + 1] = 2.52 \pm 0.25\)) than those without U. urealyticum (1.49 \(\pm 0.20, p = 0.002\)) or controls (1.31 \(\pm 0.19, p = 0.002\)). Leukocytospermia was detected in only 1 of the 17 (6%) positive specimens and 4 (12%) negative specimens.

Conclusions: Seminal ROS levels are elevated among patients with U. urealyticum. ROS induces lipid peroxidation, which reduces membrane fluidity and sperm fertilization capability, and may be the mechanism by which U. urealyticum impairs sperm function. Absence of leukocytospermia does not exclude U. Urealyticum.

KEY WORDS: urinary tract, ureaplasma urealyticum, infertility, reactive oxygen species, spermatozoa.

Ureaplasma urealyticum, previously known as T-strain mycoplasma, is the smallest free-living organism known. It is recognized as a commensal of the lower genitourinary tract of sexually active men and women but it is also suspected to be a cause of clinical infections, such as urethritis, prostatitis and pelvic inflammatory disease. For example, among our patients with chronic prostatitis we have found a 57% prevalence of U. urealyticum. The prevalence of U. urealyticum is higher among infertile (42% to 95%) than fertile (23% to 26%) couples. Several abnormal semen characteristics have been observed in the presence of U. urealyticum, such as midpiece tail defects, agglutination and impaired oocyte penetrations. Because the organism lacks a cell wall, it can adhere to the sperm membrane, thereby potentially causing the gamete dysfunction.
We confirmed whether U. urealyticum was associated with abnormal semen characteristics. We compared healthy controls to a group of men with urinary tract symptoms, including some with U. urealyticum. We measured sperm concentration, motility and viability (through acrosome reaction and mannose-binding assays). We also measured oxidative stress and leukocytospermia, which have been associated with impaired sperm quality and which we considered possible mechanisms for U. urealyticum induced sperm damage.

MATERIALS AND METHODS

This study was approved by our Institutional Review Board and all participants provided informed consent. We recruited consecutive patients seeking general urological consultation for presumed chronic prostatitis at our medical center between January 1997 and March 1998. Patients had lower urinary tract symptoms for more than 3 months with the predominant feature of pain, which was consistent with chronic prostatitis (National Institutes of Health categories II and IIIa).

Of the 61 patients who initially agreed to participate 6 who had U. urealyticum declined subsequent semen analysis and were excluded from the study. The remaining 55 patients were interviewed via a comprehensive medical history that included a description of symptoms, and history of fertility and sexually transmitted diseases. Patients were excluded from study if prostatic or testicular tumors, cryptorchidism, varicocele or neurological deficits were found during genitourinary physical examination, or if semen cultures were positive for organisms other than U. urealyticum, including Mycoplasma hominis and Chlamydia trachomatis. Five patients were excluded from study because of infection with organisms other than U. urealyticum (2), varicocele (1) and cryptoorchidism (2), leaving 50 study patients. After thorough screening 21 healthy sperm donors (WHO 1992) were recruited to participate as controls.

Semen culture. For isolation of M. hominis and U. urealyticum, the specimen was transported to the laboratory in M4 transport media and immediately inoculated in a triphasic culture system. The closed flask was then incubated at 37°C for 6 days. A color change in the medium without any turbidity was a presumptive positive result. A change from yellow to orange indicated M. hominis and from yellow to red indicated U. urealyticum alone or both organisms. When the color changed the agar surface within the flask was examined for colonies of M. hominis or U. urealyticum. A negative result was indicated by the absence of color changes and colonies.

Semen collection and assessment. Semen samples were obtained by masturbation after at least 48 hours of abstinence. Most analyses were performed on 2 separate specimens from each patient. Samples were collected in sterile containers and were allowed to liquefy at 37°C for 30 minutes. Samples were analyzed for sperm concentration, motility and morphology according to WHO 1992 criteria. Semen samples were analyzed on a computer assisted semen analyzer. For each measurement a 5 µl aliquot was loaded on a counting chamber.

White blood cell (WBC) count. The presence of granulocytes in semen specimens was assessed by the Endtz test. A 20 µl volume of liquefied specimen was placed in a 2.0 ml cryogenic vial, and 20 µl phosphate buffered saline (pH 7.0) and 40 µl benzidine solution were added. The mixture was vortexed and allowed to sit at room temperature for 5 minutes. Peroxidase positive WBCs stained dark brown were counted in all 100 squares of the grid in a chamber under the bright field objective (magnification 20 X ). The results after correction for dilution were recorded as counts X 10^6/ml. Leukocytospermia was defined as the presence of 1 X 10^6 WBC/ml. or greater of semen.

Measurement of reactive oxygen species (ROS) activity. ROS production was measured by the chemiluminescence assay method, using luminol (5-amino-2,3-dihydro-1,4phthalazinedione) as the probe. Aliquots of liquefied semen were centrifuged at 1,600 rpm for 10 minutes. The sperm pellet was washed twice with phosphate buffered saline, pH 7.4, and resuspended in the same medium at a concentration of 15 to 20 X 10^6 sperm/ml. Then 10 µl 5 µM. luminol prepared in dimethyl sulfoxide were added to 400 µl of the washed and vortexed sperm suspension. We added 10 µl 5 µM luminol to 400 µL. phosphate buffered saline to serve as a
negative control. ROS levels were determined by measuring chemiluminescence with a luminometer in the
integrated mode for 15 minutes, and expressed as 104 counted photons per minute (cpm) per 20 \times 10^6 sperm.\textsuperscript{11}

**Acrosome reaction and mannose binding assays.** The acrosome reaction, a prerequisite for fertilization, was
identified with Pisum sativum agglutinin conjugated with fluorescein isothiocyanate. A highly motile population of
spermatozoa was obtained following separation via density gradient. The 4 classes of sperm identified based on the
extent of labeling were type I-bright, type II-patchy, type III-equatorial segment only and type IV-dark. Percentage
of acrosome reacted spermatozoa was determined in a given group of patients by scoring a minimum of 200 spermatozoa.

Mannose residues are hypothesized to interact with sperm surface enzymes as a part of the recognition mechanism
that leads to sperm oocyte binding. Capacitated and acrosome reacted spermatozoa express this enzyme on the
sperm surface, where it can be detected by a fluorescent labeled lectin.\textsuperscript{12} To locate the mannose binding sites
fluorescein isothiocyanate-D-mannosylate was used, which when added to samples binds with mannose binding
sites and fluoresces apple-green. Therefore, fluorescing sperm are considered viable and scored as such.

**Statistical analysis.** Semen variables, acrosome reaction values and mannose-binding values in cases with and
without U. urealyticum, and controls were compared with the Kruskal-Wallis and pairwise Wilcoxon rank-sum
tests. For other analyses we compared U. urealyticum-positive to negative cases without controls. Values of ROS
were converted to log (ROS + 1) to normalize the distribution and analysis of variance was used to compare the
groups for this measure. To determine possible diagnostic thresholds for levels of ROS and Endtz values that
 corresponded to the presence of U. urealyticum, receiver operating characteristics (ROC) curves were calculated
with 95% confidence intervals (CI). All tests were 2-tailed and significance was assessed at p <0.05. Summary
statistics are presented as mean plus or minus standard error and all calculations were performed with commercial
software.

**RESULTS**

Mean age was 38.9 ± 1.81 years for patients with U. urealyticum, 37.8 ± 1.72 for those without U. urealyticum and
30.2 ± 2.0 for controls. Patients with U. urealyticum were more likely than those without to have had sexually
transmitted diseases (46% versus 24%), although the difference was not statistically significant (p = 0.17). Sperm
concentration and motility were superior among the controls, with no significant difference between the chronic
prostatitis U. urealyticum positive or negative specimens. The results of acrosome reaction, and mannose binding
assay did not significantly differ among the 3 groups (see table).

The most striking results were the ROS levels and WBC counts. Patients with compared to those without U.
urealyticum had significantly higher mean ROS levels (2.52 ± 0.25 versus 1.49 ± 0.20, p = 0.002), and all patients
had significantly higher ROS than controls (1.31 ± 0.19, p = 0.002). However, WBC counts were not higher in
patients with than without U. urealyticum (see table). The ROC curves for ROS and WBC count are illustrated in
the figure. The area under the curve for ROS was 75.1% (95% CI 60.7 to 89.5), which indicated a relationship
between ROS levels and U. urealyticum (p <0.001). The cutoff that maximized sensitivity and specificity was 116
\times 10^4 counted photons/minute, which provided sensitivity of 83% and specificity of 79% in diagnosing U.
urealyticum in this population. In contrast, the area under the ROC curve for WBC count was 60.1% (95% CI 43.5
to 67.6), which indicated a non significant relationship (p = 0.12). The WBC count that maximized diagnostic rates
was 0.4 million, which yielded a sensitivity of 50% and specificity of 73.3%. The widely accepted definition of
leukocytospermia, \(1 \times 10^6\) WBC/ml.; yielded a sensitivity of only 11.1%.
Comparison of semen characteristics in healthy sperm donors and patients with chronic prostatitis with or without U. Urealyticum

<table>
<thead>
<tr>
<th>Sperm Characteristics</th>
<th>Mean ± SE</th>
<th>p Value*</th>
<th>p Value †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>(21)</td>
<td>U. urealyticum Neg.</td>
<td>(33)</td>
</tr>
<tr>
<td>Concentration (x 10^6 /ml.)</td>
<td>81.59 ± 9.66</td>
<td>57.10 ± 8.87</td>
<td>60.81 ± 17.52</td>
</tr>
<tr>
<td>% Motility</td>
<td>63.02 ± 4.75</td>
<td>48.33 ± 3.56</td>
<td>50.09 ± 4.62</td>
</tr>
<tr>
<td>% Normal Morphology</td>
<td>39.86 ± 3.70</td>
<td>35.21 ± 2.29</td>
<td>32.93 ± 3.12</td>
</tr>
<tr>
<td>WBC count x 10^6/ml.</td>
<td>0.02 ± 0.02</td>
<td>0.46 ± 0.26</td>
<td>0.59 ± 0.31</td>
</tr>
<tr>
<td>% Acrosome type III (%)</td>
<td>30.28 ± 4.03</td>
<td>27.31 ± 4.84</td>
<td>32.36 ± 8.79</td>
</tr>
<tr>
<td>% Mannose type III (%)</td>
<td>41.74 ± 4.55</td>
<td>34.73 ± 6.48</td>
<td>43.60 ± 9.62</td>
</tr>
<tr>
<td>Log (ROS + 1)</td>
<td>1.31 ± 0.19</td>
<td>1.49 ± 0.20</td>
<td>2.52 ± 0.25</td>
</tr>
</tbody>
</table>

* When comparing U. urealyticum-positive and negative specimens.
† When comparing U. urealyticum-positive and negative and donor specimens.

DISCUSSION

The role of U. urealyticum in male infertility has been controversial ever since Fowlkes et al observed the prevalence of the organism among infertile couples and demonstrated the adherence of U. urealyticum to sperm through electron microscopy. Since then investigators have confirmed that the organism adheres to the head and midpiece of the spermatozoa. These observations help to explain associated motion abnormalities that may impair sperm function. Although our study demonstrated differences in sperm concentration, motility and morphology, the differences between patients and controls did not reach statistical significance. Mean values for all 3 groups were within the normal range according to WHO criteria. Nevertheless, proved infertility in men also occurs in the setting of normal semen analyses, requiring further evaluation which may reveal oxidative stress. There were no differences between acrosome reaction and mannose binding assay results but because of our small sample size, we did not have the power to detect significant differences with respect to these 2 parameters.
Adherence of *U. urealyticum* to the sperm membrane may also enhance the adverse effects of the superoxide and hydrogen peroxide produced by the organism, with subsequent spermatozoan hyper production of ROS. Our study supports this hypothesis, as the *U. urealyticum* specimens had the highest levels of ROS, which were significantly higher than levels in either controls or patients without *U. urealyticum*. The *U. urealyticum* specimens had ROS levels that were similar to those of men with proved infertility observed by Kolettis et al. These findings have clinical relevance, as ROS has been shown to induce lipid peroxidation which reduces membrane fluidity, thereby impeding sperm fertilization capacity. The abnormalities associated with elevated ROS have been well established in other settings such as varicocele, spinal cord injury and vasectomy reversal. Although ROS levels were higher in patients without *U. urealyticum* than in controls, this difference may not be as clinically important as the difference observed between patients with *U. urealyticum* and controls.

Sperm membrane changes caused by *U. urealyticum* may stimulate the formation of antisperm antibodies, which are also associated with an increased risk of infertility. Antisperm antibodies have also been observed among abacterial chronic prostatitis patients, as some cases of presumed abacterial prostatitis are unidentified *U. urealyticum* infection. Pathological conditions other than infection may also cause sperm or semen abnormalities, such as those associated with the elevated ROS levels found in our chronic prostatitis *U. urealyticum*-negative group. Surprisingly, measurement of leukocytospermia, usually performed in conjunction with semen analysis, proved to be a poor screening tool for this organism. Recently, other investigators noted the unreliability of leukocytospermia levels when evaluating subfertile men with lower urinary tract infections. Although granulocytes are considered the major contributors of ROS, ROS is also generated by spermatozoa, which might explain the lack of correlation between leukocytospermia and ROS in our study.

Because our specimens were obtained from symptomatic patients with no other detectable pathogens we might assume that *U. urealyticum* was behaving as a pathogen rather than a commensal. Quantitative cultures for *U. urealyticum* might have helped to distinguish colonization from infection. This issue remains at the center of the *U. urealyticum* controversy and the fact that we did not quantify *U. urealyticum* is a limitation of our study. However, we hypothesize that colonization alone may be sufficient to harm sperm, as sperm may be exposed to *U. urealyticum* during ejaculation and potentially in the female genital tract. In support of our hypothesis, Nunez et al demonstrated significant damage to normal non infected sperm after brief exposures to *U. urealyticum*. Longer exposure might also be associated with more significant abnormalities but time of initial *U. urealyticum* exposure could not be estimated in our patients. The *U. urealyticum* positive group had more men previously diagnosed with other sexually transmitted diseases. A history of sexually transmitted disease might be a risk factor for exposure to *U. urealyticum* and sexually transmitted diseases may themselves cause the abnormal semen characteristics.

Although there is considerable evidence linking *U. urealyticum* with infertility, there are also several studies that have found no connection. Most recently, Kanakas et al found that in vitro fertilization rates were not affected by the presence of seminal *U. urealyticum*. Preparation of the semen for in vitro fertilization may have washed the *U. urealyticum* from the specimen. A higher abortion rate was also observed in their *U. urealyticum*-positive group, possibly due to maternal factors associated with *U. urealyticum* infection. This finding may also reflect the effects of abnormal ROS on sperm DNA.

### CONCLUSIONS

The relationship among leukocytospermia, infection and infertility is unclear. Although these observations are the result of a pilot study, we presently advocate culturing and treating *U. urealyticum* in patients with chronic prostatitis and infertile couples. We are strongly opposed to empirical therapy. Evaluating men and women exposed to *U. urealyticum* using advanced laboratory technology may quell the controversy surrounding this organism, leading to its recognition as a serious pathogen rather than an innocent commensal. Further research is warranted.
REFERENCES