Infection and phagocytosis: analysis in semen with transmission electron microscopy

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Chlamydia trachomatis is the most prevalent bacterial pathogen causing sexually transmitted disease in the world. Also, several mycoplasmas species have been isolated from the human genital tract. There is conflicting evidence regarding the influence of infections with C. trachomatis and mycoplasmas on male infertility, and their exact role in development of this pathology is still debated. Transmission electron microscopy can be of great help in the study of the cellular mechanisms that underlie this male infertility condition. This is especially obvious when the small size of C. trachomatis and mycoplasmas is considered. On the other hand, there is a correlation between genital infection with these bacteria and the appearance of leukocytes in semen. There is controversy on the significance of leukocytes in semen. Finally, reports regarding phagocytosis of spermatozoa by epithelial cells of the male genital tract are controversial. In this article are presented ultrastructural findings that we encountered in semen samples of infertile men infected with C. trachomatis and/or mycoplasmas: a) structural damage of spermatozoa, b) phagocytosis of damaged spermatozoa by leukocytes, c) destruction of bacteria by leukocytes, d) persistence of bacteria in leukocytes, and e) phagocytosis of damaged spermatozoa and leukocytes by epithelial cells of the genital tract.

Keywords Infection and phagocytosis; transmission electron microscopy

1. Effect of the infection with Chlamydia trachomatis and mycoplasmas on male fertility

1.1 Chlamydia trachomatis

The family Chlamydiaceae consists of two clinically important genera, Chlamydia and Chlamydophila, with three species responsible for human disease: Chlamydia trachomatis, Chlamydophila psittaci, and Chlamydophila pneumoniae [1]. C. trachomatis, as all the members of the family Chlamydiaceae, is an obligate intracellular parasite whose developmental cycle occurs within a eukaryotic host. Infection of eukaryotic host cells is initiated by the metabolically inactive and the infectious elementary bodies (EBs). Through largely unknown mechanisms, EBs attach to and induce their internalization by host cells. Within the first few hours postinfection, EBs differentiate to the larger and more pleomorphic reticulate bodies (RBs), which are metabolically active, noninfectious, and replicative. At the end of a successful developmental cycle, the cell lyases, releasing the EBs [2, 3].

C. trachomatis infections are the most prevalent sexually transmitted bacterial infections recognized throughout the world [4]. The role of C. trachomatis with regard to inducing male factor infertility is a matter of debate. Chlamydial infection could potentially exert a strong influence on male infertility, as it is the main cause of urethritis and accessory gland inflammation in men. Sequelae of ascending infections might be occlusions in the canalicular system of the genital tract, damage of the epithelial cells involved in spermatogenesis, and immunoreaction with the production of anti-sperm antibodies [5]. Also, the relationship of C. trachomatis infection with semen quality and sperm morphology is controversial [6, 7].

1.2 Mycoplasmas

Mycoplasmas are microorganisms derived from gram-positive bacteria characterized by streamlined genomes and the absence of a cell wall. Taxonomically, the lack of cell walls is used to separate mycoplasmas from other bacteria and to place them in a class named "Mollicutes". Though the trivial terms mycoplasmas or mollicutes have been used interchangeably to denote any species included in Mollicutes, the trivial names "ureaplasmata", "entomoplasmata", "mesoplasmata", "spiroplasmata", "acholeplasmata", "astereoleplasmata", and "anaeroplasmata" are routinely used for members of the corresponding genus [8].

Mycoplasmas are most unusual self-replicating bacteria, possessing very small genomes, lacking cell wall components, requiring cholesterol for membrane function and growth, and displaying genetic economy that requires a strict dependence on the host for nutrients and refuge [9]. These reduced metabolic abilities lead them to a parasitic life-style. Mycoplasmas are capable of invading target cells and persisting and replicating for extended periods intracellularly [10].

About 200 established species have already been described within the class Mollicutes, and this number continues to rise [8]. Several mycoplasmas species have been isolated from humans. For six of them: Ureaplasma urealyticum, Mycoplasma hominis, M. genitalium, M. primatum, M. spermophilum and M. penetrans, the genital tract is the main site of colonization [11]. These microorganisms can be found commensal in lower genitourinary tracts of sexually active men and women. Moreover, they cause many disorders such as nonchlamydial nongonococcal urethritis [12, 13]. The role of mycoplasmas in male infertility has been discussed controversially. For example, some authors declare that there is no evidence that U. urealyticum has a significant impact on male fertility [14], whereas others have found that there is a significant correlation between the presence of this bacterium in semen and male infertility [15].
2. Role of seminal leukocytes in men infected with *C. trachomatis* and mycoplasmas

2.1 Biological significance of leukocytes in semen

Leukocytes are present throughout the male reproductive tract, are found in most ejaculates, and are thought to play an important role in immunosurveillance and phagocytic clearance of abnormal sperm [16]. Polymorphonuclear (PMN) leukocytes are the most prevalent type of leukocyte in semen (50% to 60%), followed by macrophages/monocytes (20-30%), and T-lymphocytes (2% to 5%) [17].

Leukocytospermia is defined by the World Health Organization (WHO) as the presence of peroxidase-positive leukocytes in concentrations greater than 1x10^6/mL of semen [18]. The role of leukocytospermia in the pathogenesis of male infertility remains controversial. Whereas some authors did not observe sperm damage in the presence of leukocytospermia, others have found evidence that leukocytes are significant cofactors of male infertility [17]. When sperm morphology is considered, several studies have found that sperm morphology deteriorates as the leukocyte concentration increases, but other studies have failed to demonstrate an association between increased leukocytic concentrations in semen and increased proportions of morphologically abnormal sperm [19]. Finally, other studies have found that semen specimens with elevated concentrations of leukocytes contained a significantly higher frequency of sperm with ideal morphology than semen specimens with low numbers of leukocytes [20].

2.2 Interaction of leukocytes with *C. trachomatis* in semen

Although *C. trachomatis* is an obligate intracellular bacterium, there is an intense inflammatory response to infection with this organism. In the initial phase, the cellular component of this response consists primarily of PMN leukocytes and macrophages [21]. Four types of *C. trachomatis*-leukocytes interaction were observed *in vitro*: (i) minimal to no bacterial binding, (ii) bacterial binding, followed by ingestion and high-level multiplication, (iii) bacterial binding, followed by ingestion but minimal multiplication, and (iv) bacterial binding, but minimal entrance or replication [22].

The genital tract infection with *C. trachomatis* has been associated with leukocytospermia. A study found both significantly higher seminal leukocyte concentrations and a greater prevalence of leukocytospermia in men who were positive for *C. trachomatis* [23]. However, other authors found no difference in *C. trachomatis* prevalence between patients with or without leukocytospermia [24].

2.3 Interaction of leukocytes with mycoplasmas in semen

Despite the vast amount of research, the mechanisms that control hosts' resistance and susceptibility to mycoplasmas infection remains unclear. Phagocytic cells, such as macrophages and PMN leukocytes, comprise the first line of defense against mycoplasmas invading the lung and genitourinary tract. Not only do they carry out effector functions such as receptor-mediated phagocytosis, but they are also involved in antigen presentation and the production of cytokines [25]. However, several studies found that leukocytes have difficulties to kill certain species of mycoplasmas [26, 27].

Most of the previous studies have reported that the presence of mycoplasmas in sperm specimens has no real effect on the leukocyte count [28, 29].

3. Transmission electron microscopy in the analysis of semen containing *C. trachomatis* and mycoplasmas

Transmission electron microscopy can be of great help in the study of infections caused by *C. trachomatis* and mycoplasmas. This is especially obvious when the small size of these bacteria is considered. *Chlamydia* species are so small, that were once considered to be viruses. Their EBs have a size of just 200-300 nm [30]. Mycoplasmas can be very small; the diameters of some viable cells are in the range of 300 nm. In fact, *M. genitalium* is considered the smallest self-replicating cell, with a genome consisting of 580 Kpb, and is theorized to approximate the essential complement of genes necessary to sustain life [31].

Previous electron microscopic studies have been mainly focused in the interaction of sperm with *C. trachomatis* and mycoplasmas. Attachment of *C. trachomatis* to human spermatozoa has been observed both *in vitro* and *in vivo* [32]. Also, ultrastructural observations indicate a physical association between mycoplasmas and spermatozoa in infected men [33]. These data suggest that spermatozoa are active agents in the dissemination of the bacterial infections to female partners, causing inflammatory processes and promoting the generation of antisperm antibodies and perhaps infertility [34]. Furthermore, it is probably that the association between the microorganisms and spermatozoa contribute to a decreased motility and necrospermia [35].
4. Phagocytosis of spermatozoa

4.1 Spermatophagy in semen by leukocytes

Non-sperm cells in ejaculates of fertile men have been studied by transmission electron microscopy. Normal ejaculate samples host active phagocytosis and possible macrophage activation [36]. In patients with accessory gland infections or subjects who have sperm antibodies in their semen, the presence of macrophages with phagocytic activity on ejaculated spermatozoa is significant [37]. Tomlinson et al. identified three types of seminal phagocytic cells: small PMN leukocytes, monocytes of similar size, and much larger macrophages capable of engulfing multiple sperm heads. Ejaculates with >50% morphologically normal forms contained significantly larger leukocyte populations. These authors suggested that the quality of sperm morphology is directly correlated with the size of the seminal leukocyte population [16]. However, other authors reported a significant positive correlation between leukocyte counts in semen and sperm tail defects, acrosomal damage and high sperm deformity index [19].

4.2 Spermatophagy by epithelial cells of the male genital tract

A long-standing question is whether phagocytosis by epithelial cells of the male genital tract acts as a quality-control mechanism to prevent defective spermatozoa from entering the ejaculate. Phagocytosed sperm in various stages of degeneration were found in the epithelial cells lining the rete testis and in the nonciliated cells of the efferent ductules in the bull [38]. Sperm phagocytosis involving the principal cells that line the cauda epididymis region in the stallion was observed [39]. Also, it has been revealed that epithelial cells of the vas deferens in the cat are extensively and actively involved in phagocytosis of spermatozoa [40].

However, some reports of sperm within epithelial cells in the normal male tract, mainly in the rete testis-efferent ductules and vas deferens-urethral junctions, amount to very small numbers indeed [41], and it has been claimed that the balance of evidence does not support the view that, in the normal animal, the epididymis routinely absorbs significant numbers of defective or dead spermatozoa [42].

5. The findings encountered by our group

In this article is presented just a minimal part of our work developed for more than twenty years in andrology research. Findings encountered in semen samples from men with infertility and subfertility of unknown cause infected with *C. trachomatis* and mycoplasmas are presented. Clinical and demographic data about the studied individuals, and also most of the experimental procedures used, have been described in a previous paper [43]. The ultrastructural images presented here complement the previous ones presented in [43], and in a recently published article [44]. Furthermore, data about leukocytospermia in these samples are now incorporated.

Briefly, 143 male partners from couples with infertility or subfertility of unknown cause, and also 10 healthy and fertile volunteers, were included in this study. The detection of *C. trachomatis* was performed in urethral samples using a direct specimen test kit (MicroTrak; Trinity Biotech, Wicklow, Ireland). The isolation of *M. hominis* and *U. urealyticum* from semen was done using the Mycoplasma IST kit (BioMerieux, Marcy L’etoile, France). The protocol used for leukocyte count in semen was adapted from Endtz [45]. Finally, ejaculates were analyzed using an electron microscopic technique.

According to the results of the microbiological tests four groups of patients were defined: 16 (11%) patients positive for *C. trachomatis*, 62 (43%) positive for mycoplasmas, 35 (25%) positive for *C. trachomatis* and mycoplasmas, and 30 (21%) negative for the two analyzed bacteria. All control subjects had negative results on microbiological tests, none presented leukocytospermia, and in only 1 of them leukocytes were found. Only 5 of the 143 patients presented leukocytospermia, and in none of the 4 groups of patients the means of leukocytes/mL of semen reached the threshold value for this condition (>1x10⁶/mL). Thus, we classified the patients as positives or negatives for the presence of leukocytes. These cells were found in 61 (43%) of the patients. In 44 of them, at least one of the studied microorganisms was detected. The findings about bacteria and leukocytes in the analyzed patients are summarized in Table 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of patients</th>
<th>No. with leukocytes</th>
</tr>
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<tbody>
<tr>
<td><em>C. trachomatis</em></td>
<td>16 (11%)</td>
<td>7 (44%)</td>
</tr>
<tr>
<td>Mycoplasmas</td>
<td>62 (43%)</td>
<td>22 (35%)</td>
</tr>
<tr>
<td><em>C. trachomatis</em> + mycoplasmas</td>
<td>35 (25%)</td>
<td>15 (43%)</td>
</tr>
<tr>
<td>Negative</td>
<td>30 (21%)</td>
<td>17 (57%)</td>
</tr>
<tr>
<td>Total</td>
<td>143 (100%)</td>
<td>61 (43%)</td>
</tr>
</tbody>
</table>
The findings observed by ultrastructure in the semen samples of the patient group included: a) structural damage of spermatozoa, b) phagocytosis of damaged spermatozoa by leukocytes, c) destruction of bacteria by leukocytes, d) persistence of bacteria in leukocytes, and e) phagocytosis of damaged spermatozoa and leukocytes by epithelial cells of the genital tract.

Many sperm head abnormalities were evident (Fig. 1): round heads, large heads, small heads and elongated heads. Vacuoles within the chromatin and a pattern of a loose fibrillar-microgranular chromatin network were also observed. The acrosome presented protuberances or was detached in most of the sperm cells observed. Other less frequently observed features were spermatozoa with disrupted nuclear membranes and cytoplasmic residues. Finally, a physical association between spermatozoa and bacteria was present in several semen samples.

![Fig. 1 Sperm abnormalities observed in semen samples of infertile or subfertile men infected with C. trachomatis and mycoplasmas. Round heads (RH), large heads (LH), vacuoles inside the chromatin (arrow), acrosomes with protuberances (double arrows), partially disrupted nuclear membrane (arrow head), spermatozoon with a pattern of a loose fibrillar-microgranular chromatin network (asterisk). Magnification: 3,000x.](image1)

Damaged spermatozoa were phagocytized by leukocytes. Also, bacteria were found degraded or localized intact in inclusion bodies into leukocytes (Figs. 2 and 3).

![Fig. 2 Ultrastructural features in semen samples of infertile or subfertile men infected with C. trachomatis and mycoplasmas. Colocalization of the two kinds of bacteria was observed in inclusions bodies of leukocytes (arrows). Leukocytes were observed phagocytizing the head (arrow head) and segments of the tail (double arrows) of sperm cells. The phagocytized spermatozoa presented several kinds of damage, like a pattern of a loose fibrillar-microgranular chromatin network (arrow head). Magnification: 3,000x.](image2)
Damaged spermatozoa and leukocytes were phagocytized by epithelial cells of the genital tract (Fig. 4).

6. Discussion

There is controversy on the role of leukocytes in the genital infections with *C. trachomatis* and mycoplasmas. In this and previous articles [43, 44], we described the ultrastructural features observed in semen samples of men with infertility and subfertility of unknown cause infected with these bacteria. The findings observed included destruction or persistence of bacteria in leukocytes, phagocytosis of damaged spermatozoa by leukocytes, and structural damage of spermatozoa. This damage would be caused directly by the microorganisms or alternatively by the host immune response.
responses [32, 46]. Thus, leukocytes may have both beneficial and detrimental effects in men with genitourinary infections with *C. trachomatis* and mycoplasmas.

However, we found that less than 1% of the samples analyzed presented leukocytospermia. This condition is defined by the WHO as the presence of peroxidase-positive leukocytes in concentrations greater than 1x10^6/mL of semen [18]. Several studies suggest that the threshold value for leukocytospermia defined by the WHO may be too high. Punab *et al.* demonstrated that the WHO-defined leukocytes cut-off point has very low sensitivity for discriminating between patients with and without significant bacteriospermia, as a more optimal sensitivity/specificity ratio appears at 0.2x10^5/mL of semen [47]. Sharma *et al.* were unable to identify a safe lower limit for leukocytes count in semen because the presence of any leukocytes, no matter how few, was associated with elevated oxidative stress [48]. Aitken *et al.* concluded that low concentrations of leukocytes in the human ejaculate caused reactive oxygen species (ROS) generation and sperm damage [49]. Henkel *et al.* found that leukocyte counts <1x10^6/mL caused a significant decrease of motility and DNA integrity of spermatozoa [50]. Thus, our data and those of others suggest that leukocytes might have a biological impact in fertility in some patients regardless of their count in semen.

It is interesting the finding that leukocytes were found in 17 of 30 patients negatives for *C. trachomatis* and mycoplasmas. Release of leukocytes may be stimulated by nonspecific male genital tract inflammation caused at first by bacteria but then continued in their absence by continued immunological activity [48]. It is possible that such patients were infected previously to their participation in the study. Another possibility is that were infected with microorganisms other than the analyzed bacteria. A follow-up with microbiological tests is necessary for them, including the use of the polymerase chain reaction technique, which is the most reliable method for detect pathogenic bacteria in sexually transmitted diseases [51]. Furthermore, until 80% of samples with the presence of leukocytes may be microbiologically negative [52].

As professional phagocytic cells, PMN leukocytes and macrophages are responsible for the first host defense mechanism during microbial infections and for regulation of other cells participating in immunological defense processes. We found in the analyzed semen samples that *C. trachomatis* and mycoplasmas were phagocyted and killed by these cells. However, localization of intact bacteria in cell inclusions was also observed. This intracellular location would results in several pathogenic consequences, like the establishment of latent or chronic infections and the dissemination of the bacterial infections to the female partners. On the other hand, our data support the hypothesis that leukocytes have a role in the removal of abnormal spermatozoa from the ejaculate. Most of the types of abnormal spermatozoa observed were also represented in the spermatophagy encountered.

Finally, we found ultrastructural evidence of phagocytosis of spermatozoa and leukocytes by epithelial cells of the genital tract in semen samples coming from subjects of the patient group. The spermatozoa observed inside the epithelial cells presented structural damage. Thus, a possible role of this phenomenon includes the clearing of degenerate or abnormal spermatozoa. Previously, we demonstrated that these epithelial cells can also phagocitize PMN leukocytes with intact bacteria inside them, and thus act in the control of the infectious process [44]. Here, we demonstrated that the epithelial cells are able of eliminate lymphocytes, a finding for which we have no a possible explanation. More research is necessary to elucidate this and other related questions about the role of the phagocytosis carried out by the epithelial cells of the male genital tract.

### 7. Conclusions

We have reported data regarding to leukocyte count in semen samples of men with infertility or subfertility infected with *C. trachomatis* and mycoplasmas. Although leukocytospermia was not significantly represented in this patient group, ultrastructural data indicated that leukocytes might have a role in fertility of some individuals infected with these bacteria. The WHO threshold value for leukocytospermia has seriously been questioned by our data and those of others and should be re-evaluated.

Taken together, our ultrastructural data suggest that PMN leukocytes and macrophages might have both beneficial and detrimental effects in male patients with genitourinary infections with *C. trachomatis* and mycoplasmas. In the former, are included the phagocytosis and the degradation of bacteria and abnormal spermatozoa. The detrimental effects include the intracellular persistence of bacteria in leukocytes and the damage of spermatozoa induced by these cells.

We reported for the first time phagocytosis of spermatozoa and leukocytes by epithelial cells of the male genital tract in semen samples. More research is necessary to evaluate the importance of this phenomenon on male fertility and in immune mechanisms present in male genitourinary infections.

Our findings demonstrate the usefulness of transmission electron microscopy in the analysis of semen samples of infertile men.
References


