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ORIGINAL ARTICLE

Transient exposure to *Chlamydia trachomatis* can induce alteration of sperm function which cannot be stopped by sperm washing

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Abstract Previous experiments have shown that *Chlamydia trachomatis* can directly affect sperm function and therefore may be an unrecognized contributor to male sub-fertility. However, the precise mechanism of sperm–chlamydial interaction remains unknown.

Aim: This experimental study attempted to replicate a transient exposure of sperm to *C. trachomatis* that might occur prior to assisted conception.

Methods: Six ejaculates from sperm donors were spiked with *C. trachomatis* serovar E and subjected to density centrifugation one hour later using a standard sperm washing technique. At 0, 6, and 24 h post-wash, the recovered motile suspension was evaluated for sperm motility, viability, phosphatidylserine externalization, DNA fragmentation and tyrosine phosphorylation.

Results: The results show that even after a relatively short exposure to *C. trachomatis*, changes in sperm motility, viability, phosphatidylserine externalization and sperm DNA fragmentation were detected up to 24 h later. Only tyrosine phosphorylation was unaffected.

Conclusion: These results suggest that sperm washing cannot protect sperm from the deleterious effects of *C. trachomatis* exposure and this may explain some cases of poor IVF outcome or fertilization failure. We suggest that all patients should be screened and treated for *C. trachomatis* prior to assisted conception. This is one of the current guidelines also recommended by the British Fertility Society.

1. Introduction

Chlamydia trachomatis is considered the most common cause of sexually transmitted diseases in men and women (1). However, whilst the effects of the organism on female fertility are well described, any effects on male fertility are more controversial (2,3).

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In vitro experiments have shown that exposure of sperm to the bacterium during co-culture can significantly alter sperm function. For example, in the presence of elementary bodies, sperms die prematurely (4) and this is associated with an increase in the tyrosine phosphorylation of sperm protein (5) externalization of phosphatidylserine (PS), and an increase in sperm DNA fragmentation associated with apoptosis (6). These effects may be mediated by the physical adherence of the bacterium to sperm as observed by electron microscopy (7–9) or by exposure to lipopolysaccharide (3,10,11).

These data have led to the suggestion that changes to sperm function as a consequence of exposure to *C. trachomatis* may contribute to male infertility. For example, if sperms encounter elementary bodies prior to ejaculation, then this may explain why the ejaculates of men contain greater numbers of dead sperms (12–14) or sperms with fragmented DNA (15) compared to their uninfected counterparts. Conversely, if sperms encounter elementary bodies during ejaculation (i.e. in the cases of lower genital tract infection of the urethra) then sperm might act as a vector to transmit chlamydia infection to the female (8) or the bacterium might contaminate the IVF culture system and be an unrecognized contributor to fertilization failure (2,3). Whilst sperm washing is now a routine part of sperm preparation prior to assisted conception, recent evidence suggests that it is not wholly effective at removing *C. trachomatis* from contaminated semen samples (14).

To examine the issue further, this paper describes an experiment in which ejaculates of donor semen were experimentally inoculated with elementary bodies of *C. trachomatis* for one hour, before sperm washing to isolate motile sperms and remove as many of the bacteria as possible. The recovered sperms were then incubated for 24 h and were assessed for sperm motility, viability, phosphatidylserine externalization, DNA fragmentation and tyrosine phosphorylation to examine how this transient exposure to elementary bodies might affect these aspects of sperm function. One hour was chosen as a typical time-window during which ejaculated sperm produced for assisted conception might be allowed to liquefy, examined by semen analysis and then be prepared for assisted conception using density centrifugation.

2. Materials and methods

2.1. Semen samples

Semen samples were obtained from six donors attending the Andrology Laboratory (Jessop Wing, Royal Hallamshire Hospital, Sheffield, UK). All donors were screened and shown to be free from sexually transmitted diseases, including HIV, in accordance with British Andrology Society guidelines (16). The ejaculates of these donors had a high sperm concentration ($>60\times10^6$ sperms/ml), with >14% ideal morphological forms (17) and no evidence of anti-sperm antibodies. The donors were of proven fertility either with their own partner or through the use of their cryopreserved semen in donor insemination treatment cycles. Each sample was produced on site after 3–5 days of sexual abstinence and collected by masturbation into a sterile wide-mouthed container (Sarstedt, Leicester, UK). Informed consent was obtained from all participants and the South Sheffield Ethics Committee reviewed all procedures and granted ethical approval (06/Q2305/21).

Samples from donors were checked to be negative for *C. trachomatis* using real-time PCR Artus *C. trachomatis* TM PCR Kit (Artus Biotech, Hamburg, Germany) (14).

2.2. Growth and preparation of *C. trachomatis* Elementary Bodies (EBs)

C. trachomatis serovar E was isolated from a clinical sample obtained from the Department of Genitourinary Medicine, Royal Hallamshire Hospital, Sheffield, UK. Confirmation of the genotype was conducted by sequence analysis according to the method of Dean et al. (18). This serovar was chosen because it has been reported to be the most frequently encountered serovar causing *C. trachomatis* infections (19). The bacteria were grown in flasks and elementary bodies (EBs) were harvested, purified and kept at −80 °C as described previously (20). Briefly, infected McCoy cells were detached from the tissue culture flask using a cell scraper (Corning Coster Co., Acton, USA) and the cell suspension was then subjected to three bursts sonication (30 s at amplitude of 12 microns) interrupted by 1 min intervals under a laminar flow cabinet. The suspension was then centrifuged at $500\times g$ for 15 min to remove cellular debris, the supernatant was then transferred to a high speed Oakridge centrifuge tube (SLS, Nottingham, UK) and centrifuged at $28{,}000\times g$ for 1 h at 4 °C to pellet the EBs. The resulting pellet was then re-suspended in 1 ml of PBS and used in the co-incubation experiment with fresh semen with the desired Multiplicity of Infection (MOI) (21). Uninfected McCoy cells were prepared and processed in the same way and used to inoculate semen samples as a (mock infection) control (see below).

2.3. Co-incubation and density centrifugation

After semen samples had been allowed to liquefy for 30 min at 37 °C, each was then divided into three 0.5 ml aliquots to which an equal volume of either: (1) chlamydial load at an MOI of 1.0; (2) uninfected McCoy cells (mock infection); and (3) Phosphate-buffered saline (PBS) were added. The aliquots were then incubated at 37 °C in a CO_2 incubator with mild shaking for one hour. All semen samples were then washed using the density centrifugation method as described previously by Nicholson et al. (22), and used in our earlier paper (14).

Briefly, 1 ml of liquefied semen was placed on the top of two 1 ml layers of 80% and 40% (v/v) of Cook Sperm Gradient Medium (Cook, Brisbane, Australia) in a sterile conical tube (Becton Dickinson Co, NJ, USA). The gradients were centrifuged at $300\times g$ for 20 min at 37 °C in a Sigma 3K18 centrifuge (Philip Harris, Ashby de la Zouch, UK). After centrifugation, the sperm pellet was recovered by first using a sterile glass Pasteur pipette to remove the top layers of the semen sample and sperm gradient, leaving approximately 0.5 ml of the bottom layer. The sperm pellet was subsequently re-suspended and washed twice in 3 ml of Cook Fertilization Medium (Cook, Brisbane, Australia) by centrifugation for 5 min at $300\times g$. The final sperm pellet was then re-suspended in a small amount of fertilization medium to give a total volume of 0.3 ml. Washed sperm suspensions with approximate sperm concentration of 10×10^6 sperm/ml were incubated at 37 °C in a CO_2 incubator for 24 h and the following tests of sperm