Experimental *Chlamydia trachomatis* infection causes apoptosis in human sperm

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BACKGROUND: *Chlamydia trachomatis* is responsible for a widespread sexually transmitted infection. In men, it is associated with a wide clinical spectrum causing infertility. Furthermore, *C. trachomatis* serovar E infection decreases motility and increases the number of non-viable sperm. No other effects of *C. trachomatis* have been reported on sperm despite the crucial role of DNA integrity for sperm function. The aim of this study was to investigate the effects of *C. trachomatis* on sperm apoptosis. METHODS: Sperm from eight normozoospermic men were incubated with increasing concentrations of *C. trachomatis* serovar E elementary bodies (EB) for 6 and 24 h. Sperm were then collected to evaluate phosphatidylserine (PS) membrane translocation and DNA fragmentation by Annexin V–propidium iodide staining, TUNEL assay and flow cytometry. RESULTS: After 6 h of incubation, *C. trachomatis* had no effect on the percentage of sperm showing PS externalization. However, a significant effect on this parameter was observed after 24 h. *C. trachomatis* also significantly increased the number of sperm with DNA fragmentation both after 6 and 24 h of incubation. CONCLUSIONS: *C. trachomatis* causes sperm PS externalization and DNA fragmentation. These effects may explain the negative direct impact of *C. trachomatis* infection on sperm fertilizing ability.

Key words: aproptesis/chlamydia trachomatis; DNA fragmentation/human sperm/phosphatidylserine membrane translocation

Introduction

Chlamydia trachomatis, an obligate intracellular parasite, has a biphasic life cycle characterized by an elementary body (EB) with infective capacity and a reticular body that is able to replicate within eukaryotic cells. *C. trachomatis* serotypes D–K are a common aetiological factor of cervicitis. It has been estimated that ~20% of women with lower genital tract chlamydial infection will develop pelvic inflammatory disease (PID), ~4% develop chronic pelvic pain, 3% infertility, and 2% adverse pregnancy outcome. Research on the effects of *C. trachomatis* infection on male infertility are much more limited (Paavonen and Eggert-Kruse, 1999). *C. trachomatis* is the recognized aetiological factor of ~50% of non-gonococcal urethritis and of the vast majority of post-gonococcal urethritis (Oriel, 1992). In men, chlamydial infection is associated with epididymitis and/or prostatitis that can lead to stenosis of the duct system, orchitis or an impairment of male sexual accessory gland function. Recent evidence suggest that it may cause male infertility by acting directly on sperm. Indeed, incubation with *C. trachomatis* has been reported to decrease sperm motility and to increase the number of non-viable sperm (Hosseinzadeh *et al.*, 2001). In addition, the presence of IgA against *C. trachomatis* correlates positively with lipid peroxidation of the sperm membrane (Segnini *et al.*, 2003). No other effects of *C. trachomatis* exposure have been reported on sperm, despite the recently highlighted relevance of sperm apoptosis on the spermatozoon fertilizing competence. Indeed, sperm DNA integrity has been associated with male infertility potential *in vivo* and *in vitro* (Zini *et al.*, 2001). Moreover, many authors reported that couples in whom the husband's semen exhibit a high percentage of sperm with DNA damage have a very low potential for natural fertility (Evenson *et al.*, 1999; Spanò *et al.*, 2000). Therefore, the aim of this study was to investigate the effects of *C. trachomatis* on sperm apoptosis. To accomplish this, sperm of normozoospermic men were incubated with EB of *C. trachomatis* serovars E; and membrane phosphatidylserine (PS) externalization, an early molecular event of apoptosis, and DNA fragmentation, a late sign of apoptosis, were evaluated by cytofluorimetric analysis.

Materials and methods

C. trachomatis *EB production*

C. trachomatis serovar E (ATCC VR-348B) EB were prepared by infecting McCoy cells, a human and murine synovia continuous cell line [American type culture collection (ATCC), CRL-1696], as previously reported (Black, 1997). Briefly, cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing glucose (0.056 mol/l), 10% fetal calf serum (FCS), gentamycin (50 µg/ml), streptomycin

(100 μg/ml) and amphotericin B (25 μg/ml), to prevent bacterial and/ or mycotic infections, up to their semi-confluence. Cells were then infected with serovar E EB in 2-sucrose phosphate buffer (2SP) by centrifugation at 750 *g* at 32°C for 1 h, according to the instructions of ATCC, and afterwards incubated in DMEM containing 2% FCS and cycloheximide (1 μg/ml) to inhibit McCoy cell protein synthesis.

Forty-eight to 72 h after the infection, cells underwent three freezing– thawing cycles, were scraped from the flasks, and sonicated three times at an intensity of 60 (Ultrasonic 2000 ARTEK with Titanium standard $^3/_4$ inch/19 mm Tip) for 30 s at 1 min interval. The suspension was then centrifuged at 500 *g* for 10 min at 4°C and the supernatant centrifuged at 30000 *g* for 20 min at 4°C. The pellet made up of semi-purified EB was resuspended in 2SP and stored at –80°C (Wang *et al.*, 1979; Yong *et al.*, 1979). Serial dilutions of the suspension obtained were used for EB quantification using shell-vials of McCoy cells which were fixed and stained with monoclonal antibody– fluorescein isothiocyanate anti-major outer membrane proteins of Micro Trak *C. trachomatis* direct specimen test (Trinity Biotech plc, IDA Business Park, Co. Wicklow, Ireland), according to the manufacturer's instructions. The mean number of inclusion-forming units (IFU) of the single dilutions, counted at the epifluorescence microscope (Leica DMLB), was used to calculate the suspension titre expressed in IFU/ml (Kuipers *et al.*, 1995; Raulston, 1997; Hosseinzadeh *et al.*, 2003).

Sperm preparation

Human semen samples were collected by masturbation after 3–5 days of abstinence from healthy subjects with negative sperm and urethral swab cultures (including for *C. trachomatis*). Sperm parameters were evaluated according to World Health Organization (1999) criteria. Only samples with sperm concentration $\geq20\times10^6$/ml, total motility $\geq50\%$, progressive motility $\geq40\%$, normal forms $\geq30\%$, viability $\geq80\%$ and leukocyte concentration $<1\times10^6$/ml were used. The sample from each individual subject was tested separately.

After 30 min liquefaction at 37°C, motile sperm were isolated using a discontinuous Percoll gradient (45/90%). The final pellet was resuspended in Biggers–Whitten–Wittingham (BWW) medium containing 0.3% bovine serum albumin at a concentration of 17.5×10^6 sperm/ml. Aliquots containing 5.25×10^6 sperm in 300 μl were infected with 0, 1, 10 or 100 μl of EB suspension (in 2SP buffer) containing 3×10^5 IFU/ml. The final volume was brought to 500 μl by adding 100, 99, 90 or 0 μl of 2SP buffer respectively, and 100 μl of BWW. Sperm were left to incubate with EB serovar E at 37°C for 6 and 24 h. At the end of the incubation, membrane PS translocation and sperm DNA fragmentation were evaluated by Annexin V–propidium iodide (PI) assay and terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labelling (TUNEL) assay and flow cytometry.

Flow cytometry

Flow cytometry analysis was performed using the flow cytometer EPICS XL (Coulter Electronics, IL, Italy) equipped with a 488 nm argon laser as light source and three fluorescent detectors corresponding to the green colour (FL-1 detector 525 nm wavelength band), orange colour (FL-2 detector 620 nm wavelength band) and red colour (FL-3 detector 620 nm wavelength band). Twenty thousand events were measured for each sample at a flow rate of 50–100 events/s and analysed using the SISTEM II™ Software, Version 3.0. The debris was gated out, by drawing a region on the forward versus side-scatter dot-plot enclosing the population of cells of interest. The compensations and the settings were adapted according to the assay utilized.

Annexin V–PI assay

Staining with Annexin V–PI was performed using a commercial kit (Annexin V–FITC Apoptosis detection kit, Sigma Chemical). An aliquot

of cells containing $\sim0.5\times10^6$/ml was resuspended in 500 μl of binding buffer, labelled with 10 μl of Annexin V–FITC plus 20 μl of PI, incubated for 10 min in the dark, according to the manufacturer's instructions, and immediately analysed by flow cytometry. Samples were evaluated by FL-1 (Annexin V–FITC) and FL-3 (PI) detectors. The cell population of interest was gated on the basis of the forward and side-scatter properties. The different labelling patterns in the bivariate PI–Annexin V analysis identified the different cell populations where FITC negative and PI negative were designated as viable cells; FITC positive and PI negative as apoptotic cells; and FITC positive and PI positive as late apoptotic or necrotic cells.

TUNEL assay

DNA fragmentation was evaluated by attaching to the breaks dUTP, conjugated to a fluocrome, in a reaction catalysed by exogenous terminal deoxynucleotidyl transferase (TdT), as previously reported (Vicari *et al.*, 2002). Briefly, sperm were labelled with the Apoptosis Mebstain kit (Beckman Coulter, IL, Milan, Italy). To obtain a negative control, TdT was omitted from the reaction mixture. The positive control was obtained by pre-treating the sperm with 1 μg/ml deoxyribonuclease I, RNAse-free (Sigma Chemical) at 37°C for 60 min before labelling. Light-scattering and fluorescence data were obtained at a fixed gain setting in logarithmic mode. The percentage of FITC-labelled sperm was determined with the FL-1 detector of the flow cytometer.

Statistical analysis

Results are reported as mean ± SEM throughout the study. Results were analysed by one-way analysis of variance (ANOVA) followed by the Duncan's multiple range test. The software SPSS 9.0 for Windows was used for statistical evaluation (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was accepted as significantly different.

Results

Infection with *C. trachomatis* EB at the concentration of 300, 3000 or 30000 IFU had no effect on the percentage of sperm with PS translocation after 6 h of incubation (Figure 1). A significant effect on this parameter was instead observed after 24 h of incubation ($P<0.001$, ANOVA). The lowest effective
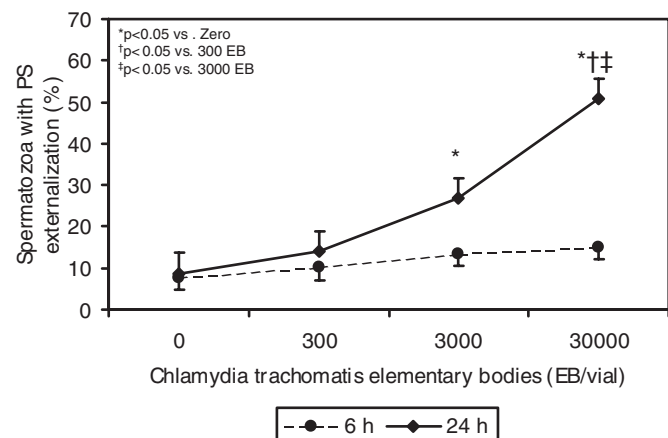

Figure 1. Effects of infection with increasing concentrations of Chlamydia trachomatis elementary bodies on membrane phosphatidylserine (PS) externalization in human sperm after 6 and 24 h of incubation. Results are expressed as mean ± SEM of eight different sperm samples.

concentration was 3000 EB which increased significantly the percentage of sperm with PS externalization compared to sperm incubated without *C. trachomatis* EB ($P < 0.05$, Duncan test). A further statistically significant increase was observed at the concentration of 30000 EB ($P < 0.05$ versus 0, 300, 3000 EB, Duncan's test).

C. trachomatis also caused a statistically significant increase in the percentage of sperm with DNA fragmentation both after 6 and 24 h of incubation (Figure 2). The effect reached statistical significance only at the highest concentration (30000 EB) of *C. trachomatis* EB ($P < 0.05$ versus 0 EB, Duncan's test) after 6 h of incubation, whereas it was effective at 3000 EB ($P < 0.05$ versus 0 and 300 EB, Duncan's test) after 24 h of incubation.

Discussion

In vitro infection with *C. trachomatis* EB induced apoptosis in sperm isolated, by a discontinuous Percoll gradient, from normozoospermic healthy men. Although EB did not have any effect on PS externalization—an early molecular sign of apoptosis—after 6 h of incubation, a significant effect was observed on DNA fragmentation with the highest concentration of EB used. After 24 h of incubation, a significantly higher number of sperm had membrane PS externalization, suggesting that a great proportion of cells had undertaken the pathway towards apoptosis. Accordingly, an elevated number of sperm showed signs of DNA fragmentation, a late apoptotic sign. Interestingly, however, a much lower percentage of sperm had DNA fragmentation compared to that externalizing PS after 24 h of incubation. This suggests that DNA fragmentation may require a longer time of exposure to become fully evident.

It is noteworthy that the pro-apoptotic effects were observed with a low number of *C. trachomatis* EB which were added at concentrations two to four orders of magnitude lower than sperm. The ability of *C. trachomatis* to cause sperm apoptosis at a low concentration and in a short span of time suggests that sperm may also be damaged during their transit in an infected female genital tract. Therefore, infection of the female partner may contribute to infertility by this additional mechanism.
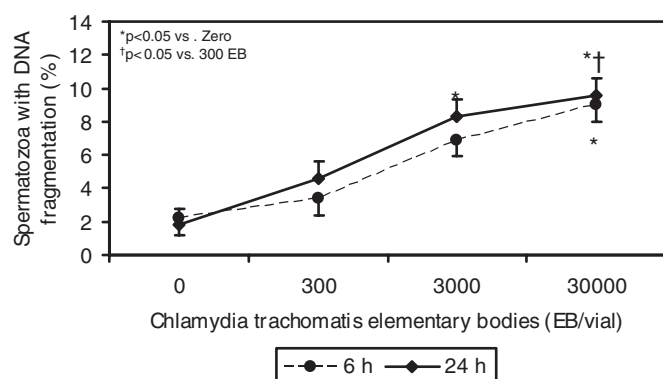

Figure 2. Effects of infection with increasing concentrations of Chlamydia trachomatis elementary bodies on DNA fragmentation in human sperm after 6 and 24 h of incubation. Results are expressed as mean ± SEM of eight different sperm samples.

Chlamydia is not the only microorganism which has been shown to cause sperm apoptosis. Recently, it has been shown that experimentally induced *E. coli* or *S. aureus* infection causes sperm apoptosis with a mechanism independent from reactive oxygen species production (Villegas *et al.*, 2005). In addition, we showed that a lack of oocyte fertilization due to an unrecognized *Candida albicans* infection was associated with an increased percentage of motile sperm with chromatin packaging abnormalities, PS externalization and DNA fragmentation (Burrello *et al.*, 2004).

The reported sperm pro-apoptotic effects of the infection with *C. trachomatis* should be added to the direct detrimental effects on other sperm parameters. Hosseinzadeh and colleagues reported that incubation with EB of serovar E (0.54×10^6/ml) resulted in a significant decline in the percentage of motile sperm and an increase in the proportion of dead sperm after 1, 3 and 6 h of incubation. Incubation with serovar LGV caused only an increase in the number of dead sperm after 6 h. The amount of acrosome-reacted sperm, the other sperm function examined, was not affected by the infection with serovar E or LGV. Dose–response experiments indicated that increasing the concentration of EB to 2.5×10^6/ml did not significantly alter the results. These effects were the result of the live organism and not of soluble components or membrane elements since they were not present after co-incubation of sperm with EB killed by heat (Hosseinzadeh *et al.*, 2001).

Incubation with *C. trachomatis* EB increased sperm protein tyrosine phosphorylation, a function which mediates important aspects of sperm capacitation and function. Indeed, it has been reported that the serovar E, but not LGV, increased tyrosine phosphorylation of proteins mainly located in the sperm tail. Immunoblotting revealed a marked increase in tyrosine phosphorylation of sperm proteins with a mol. wt of 80 and 95 kDa, whereas the serovar LGV increased phosphorylation of only the 80 kDa moiety (Hosseinzadeh *et al.*, 2000). In the attempt to establish the *C. trachomatis* EB component responsible for these effects on sperm function, the same authors showed that lipopolysaccharide (LPS) from both serovars resulted in a marked reduction in sperm motility (and a concomitant increase in the proportion of dead sperm) in a manner similar to that seen in response to EB of serovar E. In addition, when sperm were incubated with various doses of EB and LPS, the greater spermicidal effects of serovar E EB (compared with serovar LGV) were not observed when sperm were incubated with LPS from the two serovars. This suggests that the more potent effect of serovar E EB cannot be explained entirely by differences in the composition of LPS. However, that chlamydial LPS was spermicidal was demonstrated by the use of polymyxin B (a polycationic antibiotic known to neutralize LPS effects), confirming that the effects observed were primarily a result of LPS activity. Interestingly, the authors reported that *E. coli* LPS was required in doses 500 times higher than chlamydial LPS to kill a similar proportion of sperm, suggesting that bacterial LPS may differ in their spermicidal properties (Hosseinzadeh *et al.*, 2003).

The results on the effects of *Chlamydia* on sperm parameters in men are controversial. Recently, Veznik *et al.* (2004) evaluated sperm parameters of men without or with chlamydial

infection. Patients with chlamydial infection had seminal plasma volume, sperm motility, velocity and normal morphology worse than those observed in samples without infection. On the other hand, Hosseinzadeh *et al.* (2004) could not confirm that chlamydial infection was associated with any abnormality of sperm parameters. Indeed, *C. trachomatis* detected by PCR in the semen sample of 31 men (4.9% of the cases examined) was associated with significantly higher mean concentration of leukocytes and a higher mean ejaculate volume than in those whose ejaculates were PCR negative. However, there was no difference in the mean percentage motility between the two groups. The reason for this discrepancy is not clear. It may relate to the different methods (immunofluorescence versus PCR) used to detect *Chlamydia* in the semen samples and/or to the various adaptative host inflammatory responses. It is well known that PCR, being more sensitive, may have detected tiny amounts of *Chlamydia* in some samples without any negative effect on sperm parameters. Since chlamydial polysaccharide seems to be the major factor leading to sperm damage, a new investigational line has been proposed to measure its levels in the semen and to relate them with sperm parameters and sperm function (Eley *et al.*, 2005).

Our findings contribute to the understanding of the mechanisms through which *Chlamydia* may cause male infertility. Indeed, sperm from infertile men have various nuclear alterations, including abnormal chromatin structure, microdeletions, chromosomal rearrangements, aneuploidy and DNA strand breaks (Sharma *et al.*, 2004). Apoptosis is a programmed cell death based on a genetic mechanism that induces a series of cellular morphological and biochemical alterations leading the cell to suicide. In the final stage, apoptosis accounts for a significant proportion of DNA strand breaks. The extent of DNA damage is closely related to sperm function and male infertility (Aitken, 1999). In fact, intact human sperm DNA is an essential prerequisite for successful IVF and embryo development. With advanced assisted reproductive technology (i.e. IVF, ICSI), sperm selection is largely a random process that may result in the inadvertent use of DNA-damaged sperm that can fertilize an oocyte, produce high-quality early stage embryos, and then, in relationship to the extent of DNA damage, fail to produce a successful term pregnancy (Aitken *et al.*, 1999) or transmit pathology to the offspring, including childhood cancer (Aitken and Krausz, 2001).

In conclusion, these data suggest that *C. trachomatis* causes sperm PS externalization and DNA fragmentation. These effects may explain the negative impact of *C. trachomatis* infection on sperm fertilizing ability.

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