

# Co-incubation of human spermatozoa with *Chlamydia trachomatis* serovar E causes premature sperm death

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The aim of this work was to investigate the effect of elementary bodies (EB) of *Chlamydia trachomatis* serovars E and LGV on sperm motility, viability and acrosomal status. Highly motile preparations of spermatozoa from normozoospermic patients were co-incubated for 6 h with  $0.54 \times 10^6$  EB per ml. At 1, 3 and 6 h of incubation, sperm motility was determined by computer-assisted semen analysis (CASA) and the proportion of dead cells determined by the hypo-osmotic swelling (HOS) test. Acrosomal status was also examined using a standard monoclonal antibody assay. In the absence of EB, the percentage of motile spermatozoa remained >69% over the 6 h incubation and the proportion of dead spermatozoa at <12%. However, during the incubation with EB of serovar E there was a significant decline in the percentage of motile spermatozoa ( $P < 0.05$ ), and a corresponding increase in the proportion of dead spermatozoa ( $P < 0.05$ ) at all time-points. However, following incubation with serovar LGV, only the percentage of dead spermatozoa after 6 h incubation was significantly different from the control ( $P < 0.05$ ). The amount of acrosome-reacted spermatozoa remained unchanged (<16%) in all incubations at all time-points. Dose-response experiments indicated that increasing the concentration of EB to  $2.5 \times 10^6$  per ml did not significantly alter the results. Furthermore, co-incubation of spermatozoa with dead EB (killed by heat treatment) abolished the chlamydia-mediated response, indicating that the effect is a result of the live organism and not soluble components or membrane elements. These data suggest that a detrimental effect on sperm function by some serovars may be an as yet unrecognized component of infertility problems.

*Key words:* *Chlamydia trachomatis*/elementary bodies/human/serovars E and LGV/sperm function

## Introduction

Chlamydial infection is a major cause of subfertility in both males and females (Weström, 1996). The mechanism by which this occurs is thought to be via the acute inflammatory reaction associated with infection that ultimately leads to permanent scarring and functional impairment of the infected mucous membranes (Schachter, 1990). In women this is typically associated with salpingitis which can lead to the formation of lesions and the blockage of Fallopian tubes (Kossein and Brunham, 1986). 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 accessory gland function (Purvis and Christiansen, 1995). Few studies, however, have considered whether the direct exposure to *Chlamydia trachomatis* (*C. trachomatis*) itself may compromise gamete function directly and thereby lead to subfertility by a route which is independent of any damage to the reproductive epithelium.

The possibility that gametes may be directly affected by *C. trachomatis* may come about as a result of the unique

developmental cycle of the chlamydia. Briefly, the bacterium exists in two forms: alternating between an extracellular but metabolically inactive infectious form called the elementary body and an intracellular metabolically active reproducing form called the reticulate body. This is necessary because chlamydia needs to utilize the intracellular machinery of a host cell in order to reproduce. As such, the epithelium of an infected individual of either sex will periodically release elementary bodies (EB) into the reproductive tract that may then be encountered by any gametes within the reproductive tract at that time.

Previous workers have used electron microscopy to examine the possibility of interaction between human spermatozoa and chlamydial EB (Wolner-Hanssen and Mardh, 1984; Erbenji, 1993; Mavrov, 1995). However, the results of these experiments are largely inconclusive and they provide no information about the functional status of the spermatozoa. Recently, work from our group has shown that the incubation of EB from *C. trachomatis* can cause an increase in tyrosine phosphorylation of sperm proteins (Hosseinzadeh *et al.*, 2000). This is

significant because increased phosphorylation is associated with chlamydial infection of other cell types (Bliska *et al.*, 1993; Birkelund *et al.*, 1994; Fawaz *et al.*, 1997) and therefore provides indirect evidence to support the notion that chlamydia may be attaching to human spermatozoa. Moreover, it also implies that chlamydia could influence sperm function as increased phosphorylation is associated with the capacitation process (Visconti and Kopf, 1998). Interestingly, the response obtained with serovar E was greater than that observed with serovar LGV, implying that there may be differences between chlamydial serovars in terms of their infectivity of reproductive tissues and gametes.

Serovar E and LGV differ in their clinical presentation and also in the prevalence in different populations. Serovar E is more common in Europe and the USA, whereas serovar LGV is rarely seen outside the tropics and also leads to an infection with a more significant infection of the lymphatic system (Moulder, 1991; Morr e *et al.*, 2000). As such, the experiments described in this paper were performed to investigate whether the incubation of chlamydial serovars E or LGV with human spermatozoa *in vitro* could directly influence the motility patterns of human spermatozoa. Measures of viability and acrosomal status were also recorded. These were important to study, since successful fertilization requires that spermatozoa have good motility and are also acrosome intact.

## Materials and methods

Semen samples that were identified to be normozoospermic by World Health Organization (WHO) criteria (WHO, 1992) were obtained from patients attending the University Research Laboratory (Jessop Hospital, Sheffield) for diagnostic semen analysis. From each sample, a highly motile suspension of spermatozoa was obtained by density centrifugation of a 1 ml aliquot of liquefied semen through a Percoll gradient as described previously (Hosseinzadeh *et al.*, 2000). The final concentration of spermatozoa obtained was adjusted to  $\sim 20 \times 10^6$  spermatozoa/ml in Earle's balanced salt solution (EBSS) (Sigma Chemical Co., Poole, UK) containing 0.3% (w/v) human serum albumin (Sigma Chemical Co.) prior to use in the experiments described below.

Elementary bodies (EB) of *C. trachomatis* serovars E and LGV were prepared from laboratory cultures of McCoy cells that had been maintained as described by Tjiam *et al.* (Tjiam *et al.*, 1984). The culture of serovar E was initially isolated from a clinical source (cervical swab from Department of Genitourinary Medicine, Royal Hallamshire Hospital, Sheffield) and the strain LGV1 was kindly provided by M.Ward (The University of Southampton, Highfield, Southampton). Confirmation of the genotype was conducted by restriction analysis of a nested polymerase chain reaction (PCR) product of each serovar according to the method of Lan *et al.* (1994). The EB were then isolated from McCoy cells by density gradient centrifugation as described by Caldwell *et al.* (1981). Briefly, infected McCoy's cells were detached from the tissue culture flasks using a cell scraper (Costar, Corning Inc., Corning, USA) and the resulting suspension disrupted by three bursts of sonication (30 s at an amplitude of 12  $\mu$ m) interspersed by 1 min intervals. The preparation was then centrifuged at 500 g for 15 min and then transferred to a plastic centrifuge tube (Beckman Coulter Inc., Fullerton, USA) before centrifuging it again at 72 000 g for 1 h at 4°C. The resulting pellet was then resuspended in 1 ml phosphate buffered saline (PBS) and sonicated as before, then made up to a volume of 8 ml with PBS.

The chlamydia suspension was then placed on the top of 8 ml Urografin® 150 (Schering HealthCare Ltd, Burgess Hill, West Sussex, UK) in a Beckman centrifuge tube and the suspension centrifuged again as above. The final pellet was then gently washed by adding 1 ml of PBS without disturbing the pellet, before being resuspended in 2–3 ml of 2SP (2-sucrose phosphate buffer). The solution was then vortexed and sonicated as above, titrated to give a final concentration of  $1 \times 10^5$  EB and kept at  $-70^\circ\text{C}$  for further use.

### Experiment 1: 6 h incubation with chlamydia

Semen samples were obtained from 15 patients and prepared as described above to give  $3 \times 300 \mu\text{l}$  aliquots. To two aliquots,  $\sim 1.75 \times 10^5$  EB of serovar E or LGV were added in a volume of 20  $\mu\text{l}$ , giving a final concentration of EB of  $0.54 \times 10^6/\text{ml}$ . To the third aliquot an equivalent of 20  $\mu\text{l}$  of EBSS was added as a control. Each experimental tube was maintained over a 6 h incubation period in a humid atmosphere at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  in air. After 1, 3, and 6 h incubation, the tubes were gently mixed before removing two 10  $\mu\text{l}$  aliquots for the evaluation of sperm motility characteristics and sperm viability and acrosomal status respectively as described below.

### Experiment 2: dose response

Semen samples from a further seven patients were prepared to give  $4 \times 300 \mu\text{l}$  aliquots. To four aliquots 1, 2, 4 or  $8 \times 10^5$  EB of chlamydia serovars E or LGV were added in a volume of 20  $\mu\text{l}$ , giving a final concentration of EB of 0.31, 0.63, 1.25 and  $2.50 \times 10^6$  per ml. To the final aliquot 20  $\mu\text{l}$  of medium was added as a control. All incubations were maintained in a humid atmosphere at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  in air for 6 h, before removing two 10  $\mu\text{l}$  aliquots for the evaluation of sperm motility characteristics and sperm viability or acrosomal status respectively as described below.

### Experiment 3: incubation with dead chlamydia

Semen samples from a further seven patients were prepared to give  $5 \times 300 \mu\text{l}$  aliquots to which  $\sim 1.75 \times 10^5$  live or dead chlamydial EB from each serovar were added in 20  $\mu\text{l}$  of medium, giving a final concentration of EB of  $0.54 \times 10^6$  per ml. The chlamydia were killed by heating at  $56^\circ\text{C}$  for 30 min and were shown to be non-viable in subsequent cell culture. After 6 h incubation in a humid atmosphere at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ , two 10  $\mu\text{l}$  aliquots were removed from each incubation for the evaluation of sperm motility characteristics and sperm viability or acrosomal status respectively as described below.

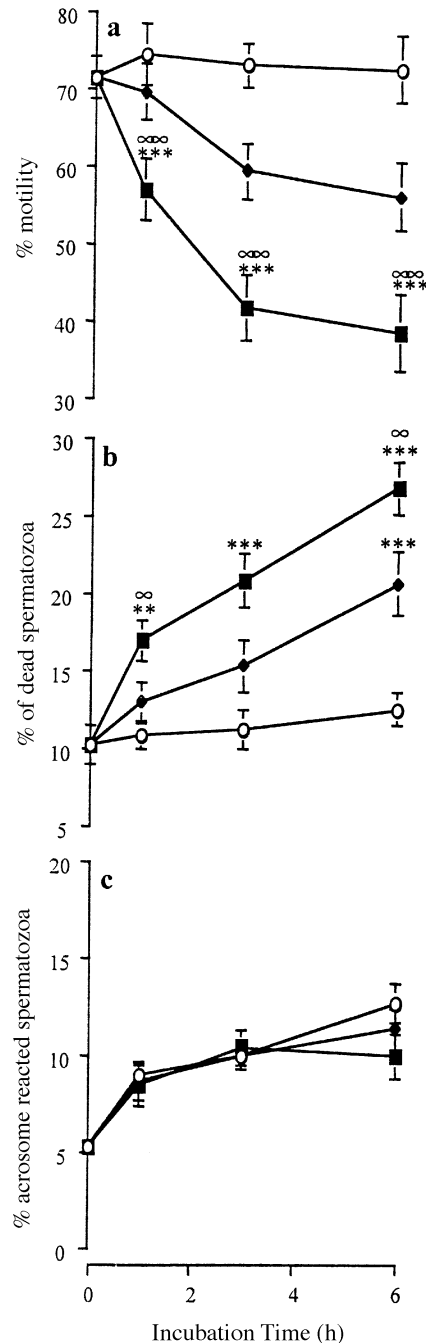
### Assessment of sperm motility characteristics

For the assessment of sperm motility, 10  $\mu\text{l}$  of the incubate was transferred into a 20  $\mu\text{m}$  depth Microcell slide (Conception Technologies, San Diego, USA). The slide was then placed on a heated stage ( $37^\circ\text{C}$ ) of an Olympus BH2 microscope® (Olympus, Tokyo, Japan) fitted with a  $\times 10$  positive phase objective and a Sony (SPT-M124) CCD camera connected to a video recorder. The videotapes were subsequently analysed using a Hamilton Thorne IVOS motility analyser (Hamilton Thorne Research, Beverley, MA, USA) running version X10.8q of the Hamilton Thorne operating system. The set-up parameters of the machine were: frame rate of 50 Hz at 50 frames/s, minimum contrast of 80, minimum size of 2 pixels, lo/hi size gates of 0.30 and 2.99, lo/hi intensity gates of 0.66 and 1.15, magnification factor 4.78.

### Assessment of sperm viability and acrosomal status

The viability of spermatozoa at each time-point was determined using the hypo-osmotic swelling (HOS) test (WHO, 1992). The HOS test was chosen as it allowed the simultaneous assessment of membrane integrity and acrosomal status to provide a simple method to determine

the percentage of live acrosome reacted spermatozoa. Briefly, 20  $\mu$ l of each incubate was transferred to 200  $\mu$ l of HOS solution (containing 0.735 g tri-sodium citrate and 1.351 g fructose in 100 ml distilled water) and incubated at 37°C for 30 min. Each incubate was then



**Figure 1.** Details of percentage sperm motility (a), viability (b) and percentage of acrosome-reacted spermatozoa (c) over a 6 h incubation period with  $0.54 \times 10^6$  chlamydial elementary bodies (EB) per ml of serovar E (■) or LGV (◆). The results of a control incubation (○) are also shown. Data shown are the mean  $\pm$  SEM of incubations with sperm preparations from 15 patients. Statistical differences were examined using a one-way analysis of variance (ANOVA) on the log-transformed data, with differences between groups examined using a Bonferroni multiple comparison test. The symbol (\*) indicates a significant difference from the control and the symbol ( $\infty$ ) indicates a significant difference from LGV. The level of significance is indicated by the number of symbols: 1 $\times$  =  $P < 0.05$ ; 2 $\times$  =  $P < 0.01$ ; and 3 $\times$  =  $P < 0.001$ .

spotted onto a microscope slide and allowed to air dry overnight, before being fixed in absolute methanol for 45 min the following day. The slides were then stained to evaluate the acrosomal status using the technique described by Moore *et al.* (Moore *et al.*, 1987). Briefly, each slide was incubated with 100  $\mu$ l of the primary monoclonal antibody for 60 min in a moist-chamber at 37°C. Prior to addition of second antibody [sheep anti-mouse immunoglobulin (IgG) fluorescein isothiocyanate conjugated] the slides were washed in PBS (Gibco, Paisley, UK), and then incubated for an additional 40 min at 37°C. The slides were finally washed in PBS and mounted using MOWIOL<sup>®</sup>4-88/1,4-diazobicyclo-(2,2,2)-octane (Ellis *et al.*, 1985). Each slide was then observed on an Olympus BH2 microscope<sup>®</sup> (Olympus) fitted with both  $\times 100$  magnification phase contrast and epifluorescence objectives (UV filter, 492 nm). For each incubate or control, 200 spermatozoa were counted to establish the viability of the sample, recording all spermatozoa with straight tails as 'dead'. A further 200 spermatozoa were examined to determine the proportion of viable (coiled tailed) spermatozoa that were acrosome intact (spermatozoa with entire fluorescence over the acrosomal region).

### Statistical analysis

All the data generated in these experiments were non-parametric; as such the results were transformed to  $\log_{10}$  to allow an analysis by a two-way analysis of variance (ANOVA).

## Results

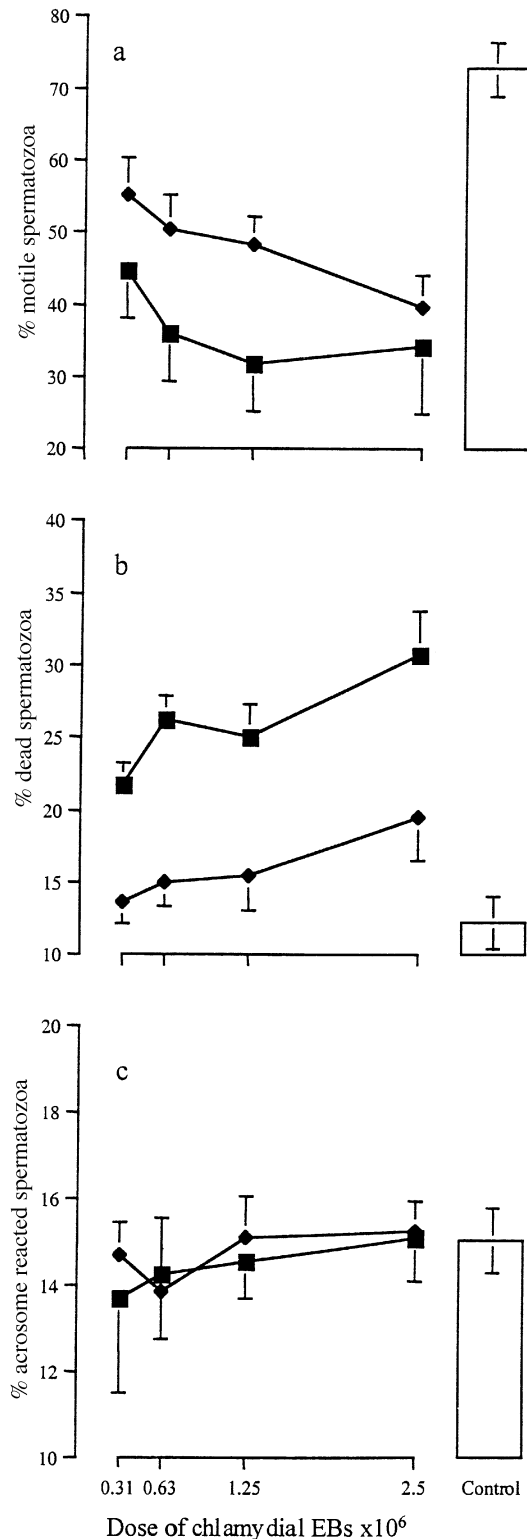
### Experiment 1

Immediately after density gradient centrifugation, the sperm preparations had a sperm motility of  $71.6 \pm 2.8\%$  (Figure 1a) with only  $10.3 \pm 1.2\%$  dead cells (Figure 1b) and  $5.3 \pm 0.4\%$  of live spermatozoa having lost their acrosomes (Figure 1c); data are mean  $\pm$  SEM for  $n = 15$  experiments. Over the 6 h incubation period, these characteristics did not alter significantly in the control population ( $P > 0.05$ ).

However, spermatozoa that were incubated with EB of chlamydial serovar E showed an almost immediate decline in the proportion of motile spermatozoa (Figure 1a). After 1 h of incubation, for example, sperm motility had fallen to  $57.1 \pm 3.9\%$  and this was significantly different from the control incubation ( $P < 0.001$ ) and the spermatozoa incubated with serovar LGV ( $P < 0.01$ ). This fell to  $41.9\% \pm 4.3$  and  $38.7\% \pm 5.1$  after 3 and 6 h incubation respectively and these values were also significantly different from the control ( $P < 0.001$ ) and LGV ( $P < 0.01$ ) incubations. Although spermatozoa incubated with serovar LGV also showed a numerical decline in the percentage of motile spermatozoa, this was not significantly different from the control values at any time-point.

Concomitant to the decline in sperm motility, an increase in the proportion of non-viable spermatozoa (as determined by the HOS test) was also observed over the 6 h incubation (Figure 1b). Interestingly, the extent of this rise in cell death was again not the same between serovars, since serovar E caused the highest amount of cell death:  $17.0 \pm 1.3\%$  of spermatozoa becoming non-viable after 1 h of incubation, rising to  $20.9 \pm 1.8\%$  and  $26.9 \pm 1.7\%$  after 3 and 6 h respectively. This was significantly higher than the control at all time-points, with  $P < 0.01$  at 1 h and  $P < 0.001$  at 3 and

6 h. Similarly, spermatozoa incubated with LGV also showed an increase in the proportion of non-viable cells, although this was only significantly higher than that observed in the control incubation after 6 h incubation ( $P < 0.001$ ) when  $20.7 \pm 2.0\%$  of spermatozoa were non-viable. After 1 and 6 h of incubation the proportion of spermatozoa incubated with LGV that were non-viable was also significantly different from that observed following incubation with E ( $P < 0.05$ ).



Interestingly, the amount of acrosome-reacted spermatozoa remained unchanged ( $<16\%$ ) in all incubations at all time-points (Figure 1c).

**Experiment 2**

After 6 h of incubation in the absence of chlamydial EB,  $72.6 \pm 10.1\%$  of spermatozoa in this experiment were motile (Figure 2a) with only  $12.0 \pm 4.7\%$  of dead spermatozoa in the populations (Figure 2b) and  $15.0 \pm 2.0\%$  of live spermatozoa being acrosome reacted (Figure 2c). After 6 h incubation with increasing concentrations of chlamydial EB, however, there was a marked decrease in the proportion of motile spermatozoa (Figure 2a) and a marked increase in the proportion of non-viable spermatozoa (Figure 2b), as would be predicted from the results in experiment 1. However, two-way analysis of variance of the motility and viability data indicated that at EB concentrations of  $0.31 \times 10^6$  EB per ml or greater there was no further effect of dose, although there was a significant difference between the serovars ( $P < 0.01$ ). Acrosomal status (Figure 2c) was independent of dose and serovar.

**Experiment 3**

Figure 3a–c shows the results of a 6 h incubation of spermatozoa with live or dead EB of serovar E and LGV. Following incubations with serovar E there was an expected significant decrease ( $P < 0.001$ ) in the proportion of motile spermatozoa when compared to the control that was not seen following incubation with dead EB (Figure 3a). In addition, there was an expected increase ( $P < 0.001$ ) in the percentage of non-viable spermatozoa following incubation with live EB (Figure 3b) but this was not seen following incubation with dead EB. Spermatozoa incubated with LGV showed only a slight, but not significant, decrease in the percentage of motile spermatozoa (Figure 3a) and a concomitant slight, but not significant, increase in the percentage of non-viable spermatozoa (Figure 3b). This response was the same irrespective of whether the EB were live or dead. Acrosomal status (Figure 3c) was unaffected by either serovar, whether live or dead.

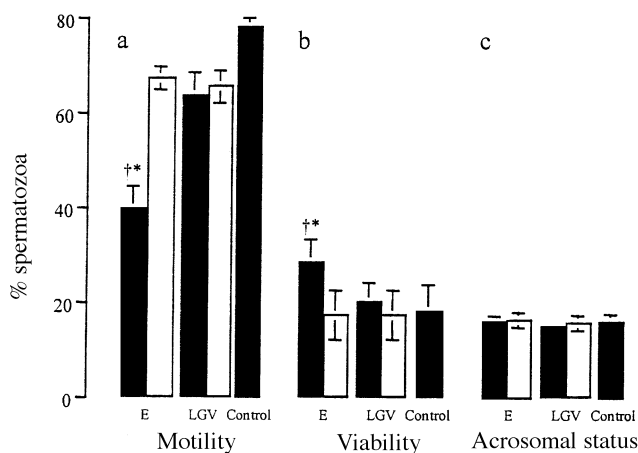
**Discussion**

It is currently controversial whether *C. trachomatis* infection has a significant effect on human sperm function. Some studies

**Figure 2.** Details of percentage sperm motility (a), viability (b) and percentage of acrosome-reacted spermatozoa (c) following a 6 h incubation period with doses of chlamydial EB of serovar E (■) or LGV (◆) ranging between  $0.31\text{--}2.5 \times 10^6$  per ml. The results of a control incubation (time = 6 h) are also shown (vertical bar). Data shown are the mean  $\pm$  SEM of incubations with sperm preparations from seven patients. Statistical differences were examined using a two-way ANOVA on the log-transformed data and indicated that at EB concentrations of  $>0.31 \times 10^6$  per ml, there was no further effect of dose, although there was a strong effect of serovar ( $P < 0.01$ ) upon sperm motility and viability. Sperm acrosomal status was unaffected by chlamydial serovar or dose.

(Custo *et al.*, 1989; Wolff *et al.*, 1991; Cengiz *et al.*, 1997) have shown that chlamydial infection is associated with reduced semen quality, whereas others (Nagy *et al.*, 1989; Eggert-Kruse *et al.*, 1990, 1996, 1997; Soffer *et al.*, 1990; Weidner *et al.*, 1996; Habermann and Krause, 1999) have found no difference between the semen quality of infected and non-infected males. Such studies are, however, difficult to interpret because of three main criticisms. First, no study takes into account the significant inter- and intra-individual variation in semen quality that is known to exist (WHO, 1992). Second, all of the studies to date have used unreliable semi-quantitative manual methods to assess semen quality that are known to be subject to large measurement errors (Dunphy *et al.*, 1989). Finally, the use of serology to determine chlamydial infection is known to be problematic and will not give accurate findings.

All of the above criticisms have been overcome by the in-vitro design employed in the experiments reported in this manuscript. First, methods of sperm preparation were used to standardize in each experimental treatment the number of motile spermatozoa ( $20 \times 10^6/\text{ml}$ ). Moreover, because semen for experimentation was only obtained from normozoospermic males, the effects of chlamydia on spermatozoa are much easier to see than they are in studies that compare the semen profiles of men with or without an infection. Second, spermatozoa were incubated with a known number of chlamydial EB from well-characterized laboratory cultures and as such, the number of bacteria could be standardized between experiments. Finally, sperm motility was assessed using modern computer assisted semen analysis (CASA) (Mortimer, 1994), which is known to provide accurate and precise data concerning the movement characteristics of sperm populations. Sperm viability was also assessed using standard techniques (WHO, 1992) and sperm acrosomal status evaluated using monoclonal antibodies developed for this purpose (Moore *et al.*, 1987).



**Figure 3.** Results from incubation of motile spermatozoa with live (■) or dead (□) EB prepared from laboratory cultures of serovar E or LGV. The results of a control incubation (no EB) are also shown. The data shown are the mean of seven experiments  $\pm$  SEM. The parameters measured are (a) sperm motility, (b) sperm viability and (c) sperm acrosomal status. The symbol (\*) indicates a significant difference from control incubation ( $P < 0.001$ ) whereas (†) indicates a significant difference from the dead serovar ( $P < 0.001$ ).

The results of the current study are clear and provide irrefutable evidence that chlamydial EB can have a direct and detrimental effect on sperm physiology. The primary route of the effect would seem to be by causing premature sperm death (loss of viability) which is reflected in a corresponding loss of sperm motility. Exactly how chlamydial EB are able to cause sperm death is currently unknown, although it is clear that the effect relies upon the presence of live EB or at least a heat labile secretion since heat treatment abolishes the effect described in this study. A previous study has proposed that a lipopolysaccharide purified from serovar LGV can cause sperm death (Galdiero *et al.*, 1994), which is interesting given that in the experiments presented here it was serovar E that had the most effect. However, this potential mechanism clearly requires further investigation.

That the incubation of spermatozoa with serovar E gave rise to a significantly greater response than LGV is an interesting observation that requires a more in-depth study. Differences between these two serovars have been noted previously and whilst both are a common cause of genital infection, LGV is also associated with infections of the lymphatic system (Moulder, 1991). Moreover, in an earlier report from our group (Hosseinzadeh, 2000), it was shown that serovar E but not LGV caused increased tyrosine phosphorylation of sperm proteins, suggesting an additional difference between the serovars and their effect on spermatozoa. Furthermore it will be important to establish in due course whether other chlamydial serovars also have a negative effect on sperm function or whether this is limited to serovar E as described here.

In our earlier work (Hosseinzadeh, 2000), it was shown that the EB-induced increase in tyrosine phosphorylation could be localized to the sperm tail region. Moreover, serovar E caused increased tyrosine phosphorylation of two major sperm epitopes of 80 and 95 kDa whereas serovar LGV only caused increased tyrosine phosphorylation of the 80 kDa epitope. As such, it was postulated that such effects may compromise sperm function by accelerating sperm capacitation, since tyrosine phosphorylation of sperm proteins is closely associated with capacitation *in vitro* (Visconti and Kopf, 1998). However, at that time it was not technically possible to assess sperm motility, but from the results presented here it now seems more likely the chlamydial-induced increase in tyrosine phosphorylation is associated with cell death rather than capacitation.

A commonly held view is that the main influence of *C. trachomatis* on male fertility is not its effect on semen quality but is based upon sexual transmission to the female with a subsequent negative influence on tubal function (Eggert-Kruse *et al.*, 1990). Indeed, it has been suggested that the male may act as a reservoir of infection and it has been proposed that spermatozoa may provide a route of chlamydial infection to the Fallopian tubes (Purvis and Christiansen, 1995; Keck *et al.*, 1998). Given the results described in this paper, however, it is clear that this mechanism may not be the same across all serovars, given that upon exposure to serovar E sperm motility is rapidly abolished.

In understanding how chlamydia may affect spermatozoa

directly it is important to consider the physiological conditions by which spermatozoa might be exposed to the organism. An important question is whether the concentration of EB used in these experiments is physiological and whether they would be the concentrations encountered by spermatozoa in either the male or the female reproductive tracts. This is not an easy question to answer, although we know from the sensitivity of the current diagnostic methods used in clinical practice that a positive sample must contain  $\geq 10^4$ – $10^5$  EB (Black, 1997). However, the exact concentration will vary according to the tissue type, the host response and the stage of developmental cycle of the organism. It is quite possible, therefore, that at the time of release of EB from a host cell, the local concentration of EB may be significant. As such, the addition of  $0.54 \times 10^6$  EB to the experimental conditions described here would seem both appropriate and physiological.

In an infected male, then, it is possible that spermatozoa may be exposed to EB during epididymal transit and storage. As such, one might predict that an ejaculate from a male with an active infection might contain increased numbers of non-motile and non-viable spermatozoa. Whether this is the case requires investigation, since the existing literature is not clear on this point. For example the papers by Eggert-Kruse *et al.* (1990, 1996, 1997) considered only past and not current chlamydial infection and the papers by Custo *et al.* (Custo *et al.*, 1989) and Soffer *et al.* (Soffer *et al.*, 1990) did not use WHO methodology in their analysis of semen. However, if the site of the chlamydial infection is in the accessory glands or the urethra, then it is possible that spermatozoa may not be exposed to EB until the time of ejaculation. In such events, the exposure time may be insufficient for the semen analysis to detect any real influence of EB on sperm motility and viability.

In an infected female, spermatozoa may become exposed to chlamydial EB at any point during their journey to the site of fertilization and if this were to occur early enough (e.g. within the cervix) then it remains possible that spermatozoa may die before they ever reach the Fallopian tubes. It has been shown that in cases where antibiotic therapy has failed, cultures of cervical mucus are positive for chlamydia up to 14 days after the completion of the therapy (Black, 1997), suggesting that EB may persistently be present in the cervix of an infected woman. Moreover, it is now known that if intercourse occurs prior to ovulation, then a reservoir of spermatozoa is probably formed in the tubal isthmus, close to the utero–tubal junction (see Pacey *et al.*, 1995a, b). As such, spermatozoa may be resident in the Fallopian tube for several days prior to ovulation and during this time they may presumably be under assault from chlamydial EB.

Within a long-term relationship it would seem likely that both of the above mechanisms might occur simultaneously, as it is unlikely that with regular intercourse both partners would remain uninfected, irrespective of which first contracted the infection. However, there are instances in assisted conception techniques where spermatozoa may become exposed to chlamydia. For example, during donor insemination (DI) spermatozoa from a screened and known chlamydia negative donor (British Andrology Society, 1999) may be inseminated into a woman in order to achieve a pregnancy. Since donor

spermatozoa are cryopreserved and generally considered more fragile than fresh spermatozoa (Keel and Webster, 1993) then it remains possible that donor spermatozoa may be more susceptible to damage by chlamydial EB than fresh spermatozoa. Furthermore, not all infertility clinics screen their patients for chlamydial infections prior to DI and so the status of the female partner at the time of treatment might be unknown. During more complex IVF procedures, donor or partner spermatozoa are sometimes incubated with oocytes under conditions not dissimilar from the experimental set-up used in this paper. Although sperm washing techniques can remove microbes from human semen (Wong *et al.*, 1986), *C. trachomatis* could be introduced into the system from the oocyte (De Punzio *et al.*, 1991) if the female partner has an active infection. Under such conditions, it is clear that spermatozoa may be introduced into an environment where they will be exposed to EB and with certain serovars may rapidly lose their motility and viability and therefore contribute to fertilization failure.

In conclusion, the results described in this manuscript provide strong evidence to suggest that an additional mechanism of chlamydial-mediated infertility may be as a result of a direct effect of chlamydial EB on spermatozoa. This concept requires further investigation to determine its cellular basis and the data would suggest that screening for chlamydial infection should become a routine part of the work-up of the infertile couple and should be included in screening procedures prior to entry to an assisted conception programme. Recommended methods of screening are by an amplification technique, such as ligase chain reaction, of urine taken from men and a cervical swab taken from women. Testing from antibodies is not recommended.

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