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Escherichia coli attaches to human spermatozoa: affecting sperm parameters

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ABSTRACT

Infection is a powerful mechanism that can lead to sperm damage, deformity and eventually, male infertility. Genital tract infection and inflammation have been associated to 8-35% of male infertility cases. E. coli causes agglutination of human spermatozoa and thus, leading to infertility. This study evaluated if the negative influence of Escherichia coli on the motility of human spermatozoa is a consequence of E. coli adhesion or some soluble factor from E. coli are involved. Highly motile preparation of spermatozoa from normozoospermic patients were obtained by 'swim up' procedure. Study was carried out in three parts. Motility parameters were analysed by light microscopy and Nomarski differential interference contrast microscopy directly, and 30 min, 2, 4, 6 h and overnight after inoculation. In a second series of experiments, bacterial replication was inhibited by addition of five antibiotics amoxicillin, ciprofloxacin, erythromycin, gentamicin and penicillin G. The effect of E. coli culture filtrates on sperm motility was investigated. In a third series, E. coli growth was suppressed by heating at high temperature (100^0 C) for 30 min. E. coli decreased the sperm motility and viability by agglutination immediately after inoculation and effect increases as the time of incubation increases. Instead of this, both the culture medium in which E. coli growth was inhibited (antibiotic and heating), was not able to cause any change in motility and viability. Further, completion of incubation at 6 h the percentage of motile spermatozoa left is 11% that is very low as compared to control. This shows that E. coli may be carrying some adhesion sites through which it binds to human spermatozoa and causes agglutination.

Keywords: *Escherichia coli*, spermagglutination, antibiotic, infertility, immobilization.

INTRODUCTION

Infection is a powerful mechanism that can lead to sperm damage, deformity and eventually, male infertility. Genital tract infection and inflammation have been associated to 8-35% of male infertility cases [1, 2]. *E. coli* is the most important pathogen causing prostatitis and epididymitis

[3-5], but is also found in semen samples of infertile men [6] without leukospermia and clinical signs of infection [6, 7].

Among the most common microorganisms involved in sexually transmitted infections, interfering with male fertility, there are the *Chlamydia trachomatis* and *Neisseria gonorrhoeae* [8]. Sperm motility may also be inhibited by direct effects of the bacteria on spermatozoa, for example bacterial adhesion [9] or cytotoxins [10], or, more indirectly, by noxious effects of the induced inflammatory process, for example polymorphonuclear leukocytes [11], macrophages and their toxic secretory products [12]. It has been found that *U. urealyticum* can attach massively to sperms, especially at the mid-piece, thus producing marked hydrodynamic drag on infested sperm [13]. Colony of *Candida albicans* can cause sperm clumping. 50% of the sperm in the specimen undergo clumping, thereby greatly reducing the effective sperm motile count [14]. *Mycoplasma genitalium* was demonstrated to adhere to all parts of the spermatozoa. Numerous *M. genitalium* cells bound to the spermatozoa were shown to cause sperm agglutination. Inhibition of sperm motility has also been documented for other human and animal *Mycoplasmas* [8, 15].

The aim of this work was to study the effect of *E. coli* on sperm motility and viability. We conducted some series of experiments in order to determine whether decrease in motility was associated with adhesion of *E. coli* to sperm or some soluble factors causes the above said changes.

MATERIALS AND METHODS

Sperm preparation and incubation

Semen samples were retrieved from 30 healthy donors with a normal sperm count (>20×10^6 spermatozoa/ml; 16). The semen samples were kept at room temperature, in darkness and for no longer than 3 h before use. The spermatozoa were purified by a `swim-up' procedure. Only ejaculates with normal sperm concentration and forward motility were used for further investigation. Spermatozoa were washed in HAM'S F-10 medium and centrifuged at 400 g for 10 min. After removing the washing solution, the pellet containing the cells was overlayed with 1 ml Ham's F-10 medium prewarmed to 37^0C and incubated for 30 min at 37^0C. After incubation, the liquid phase was isolated and stored in a sterile vial. This 'swim-up technique' was performed in all experiments to achieve a purified sperm suspension of high motility and to prevent potential antimicrobial activity of seminal plasma [17] in further investigations. This suspension was diluted to a sperm concentration of 20 million/ml with culture medium to standardize the experiment and to establish exact sperm/bacteria ratios. After dividing the suspension into five fractions, four were inoculated with a suspension of *E. coli* in Ham's F-10, and one was inoculated with sterile medium as control.

Microorganism

E. coli used in the present study was prepared from laboratory cultures that have been isolated as described by Prabha *et. al.*, 2009 [18]. The culture of *E. coli* was initially isolated from a clinical source (semen samples from patients with unexplained Infertility visiting Department of Obstetrics and Gynecology, General Hospital, Sector-16, Chandigarh).

Experimental Design

Three experiments were performed. Experiment 1: incubation was performed in Luria broth (Hi-Media Laboratories, India) medium. When motility was measured, bacterial concentration was also determined in the Cellometer™ counting chamber, to evaluate the bacterial growth in each

sample. Experiment 2: To prevent bacterial growth effectively five antibiotics were used in present study Amoxicillin, Ciprofloxacin, Erythromycin, Gentamicin and Penicillin G (Sigma Chemical Co., USA). Stock solutions (1mg/ml) of each antibiotic were prepared in sterile distilled water as recommended by the manufacturer. MBC (Minimal bactericidal concentration) for each antibiotic was obtained using the standard protocol [19]. MBC was defined as a 99.99% reduction of cell viability with respect to that of initial inoculums. In time–kill studies, the MBC is the minimal amount of antibiotic that results in a ≥99.9% decrease in the initial inoculums within 24 h in a standard test. Other conditions of incubation remained the same as in the first experiment. A total of 15 single experiments were undertaken. Filtrates of *E. coli* cultures were obtained by filtering an *E. coli* suspension grown in Luria broth for 48 h at 37^0C, through a Millipore filter (0.22 µm). This was done to establish whether a soluble factor from *E. coli* might influence sperm motility. Experiment 3: This was performed same as experiment 2 except the growth of *E. coli* was stopped by heating at 100^0 C for 30 min. After completion of incubation the absence of viability was confirmed by plating on nutrient agar plates. A highly concentrated suspension of *E. coli* served as a positive control, pure Luria broth as a negative control. Filtrates of *E. coli* cultures were mixed 1: l with sperm suspensions. Besides these investigations on motility, morphological changes in spermatozoa induced by *E. coli* were also examined. Morphological studies were undertaken using light microscopy and Nomarski differential interference contrast microscopy.

Light microscopy and Nomarski differential interference contrast microscopy (NM):
The sperm and *E. coli* were mixed 1:1 (v/v), and examined by light microscopy using × 10 objectives immediately, 30 min, 2, 4, 6 h and after overnight incubation at room temperature. For studies using NM, the sperm- *E. coli* suspension was incubated at 37°C and studied after 5 min, 30 min, 1 h, 2 h, 6 h and overnight incubation. At each time point, an aliquot of 3 µl was mounted on slides, and after a few minutes the sperm stuck to the glass coverslips. A total of 3 × 100 spermatozoa were counted and the number of sperm with *E. coli* attached was determined.

Assessment of sperm viability by Eosin-Nigrosine:
The viability of agglutinated spermatozoa at each time-point was determined using eosin-nigrosine. Briefly, two drops of 1.0% eosin were added and mixed well. After 30 sec three drops of 10% nigrosine were added and mixed. The mixture was smeared and allowed to air dry on a clean glass slide. The slide was observed under a light microscope at X100 to differentiate the unstained live cells from the pink stained dead spermatozoa. At least 20 fields were read for each time interval and average values were noted.

RESULTS

Experiment 1:
Immediately after density gradient centrifugation, the sperm preparations had a sperm motility of 73.4% (n= 30) (Graph 1) and with only 10.3% dead spermatozoa.

Purified spermatozoa were incubated with *E. coli*, and sperm motility was studied using ordinary light microscopy. The spermatozoa started to clump immediately after mixing.

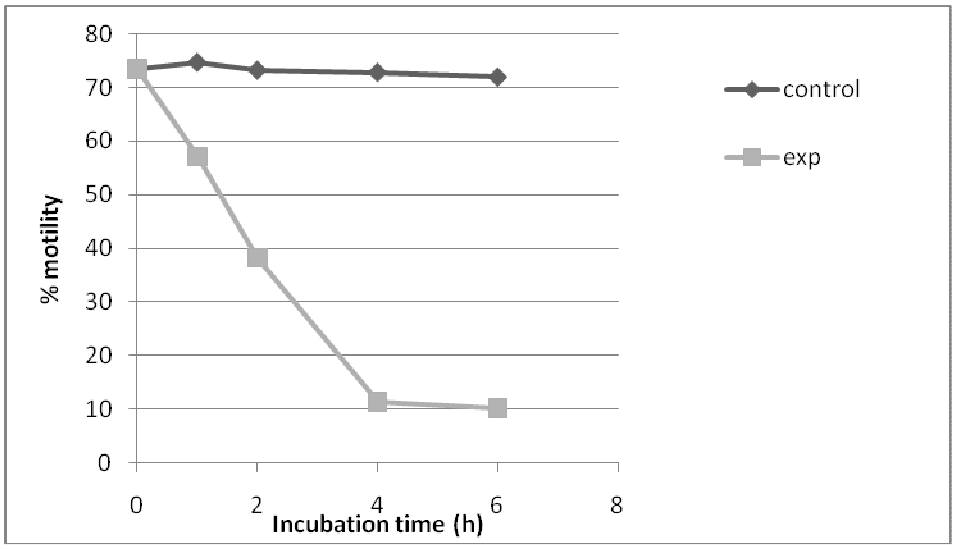
After 5 min of incubation, small sperm agglutinations could be detected and, with time, the agglutinations increased in size. Repeating the experiments showed that the sizes and number of the agglutinates varied with 10-fold dilutions of *E. coli* and sperm obtained from different donors.

After 1 h of incubation, for example, sperm motility has fallen to 57.1% and this was significantly different from the control incubation. Further completion of incubation at 6 h the percentage of motile spermatozoa left is 11% that is very low in comparison to control. Over the 6 h incubation period, these characteristics did not alter significantly in control population.

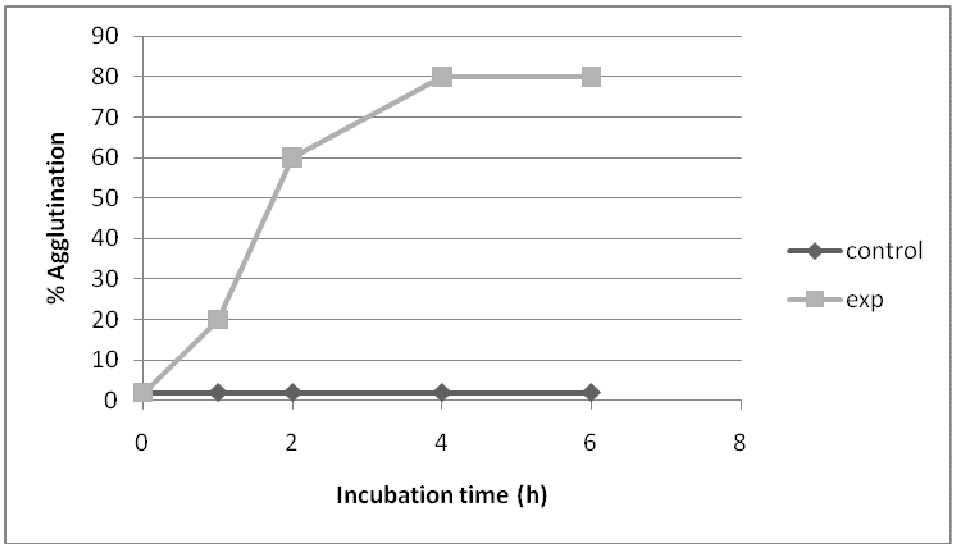
Agglutination was determined for both the samples (Graph 2). In experimental group, the agglutination of bacteria was observed while in case of control no agglutination was observed (≥ 5%).

At each time point, an aliquot of 3 µl was mounted on slides, and after a few minutes the sperm stuck to the glass cover slips. A total of 3 × 100 spermatozoa were counted and the percentage of agglutination of sperm was determined.

Graph 1: Percentage of motility of spermatozoa when incubated with live *E. coli* and control



Graph 2: Percentage agglutination of spermatozoa when incubated with live *E. coli* and control



Experiment 2: MBC for these five antibiotics i.e. Amoxicillin, Ciprofloxacin, Erythromycin, Gentamicin and Penicillin G are shown in table 1. These antimicrobial agents were used to inhibit the growth of *E. coli* and positive control was not treated with any antibiotic. High

agglutination was observed in control. In experimental group, killed bacteria failed to bring about the same change.

Table 1: MBC of antimicrobial agents

Antimicrobial	MBC(µg/ml)
Amoxycillin	15
Ciprofloxacin	33
Erythromycin	10
Gentamicin	4
Penicillin G	10

Experiment 3: The bacterial growth was stopped by heating the 48 h old culture for 30 min. Both of the culture (experiment 2 & 3) showed no growth on nutrient agar plates. Positive control was incubated at 37^0 C. The heat treated *E. coli* did not showed any changes in the sperm regarding to motility and viability. This shows the inference in motility is may be due to *E. coli* and not the soluble factor.

DISCUSSION

The present study demonstrated that human sperm motility and viability was affected by *E. coli*. *E. coli* isolated from the semen of infertile male produced profound depression in the motility of human spermatozoa by agglutination *in vitro*. Schirren & Zander (1966) [20] in past reported a negative influence of *E. coli* on sperm motility after mixing sperm and bacteria in vitro. This negative influence was confirmed by several authors [21-23, 10]. Teague *et al.* (1971) [21] were the first to claim high numbers of bacteria as causative for this effect. After addition of antibiotics to the incubation medium, all bacterial effects on sperm motility disappeared. Bacterial growth may be one cause of contradictory conclusions concerning the effective sperm/bacteria ratio. The absence of sperm agglutination was also observed after heat treatment of bacteria. In one of the earlier reports on effect of heat killed bacteria on spermatozoa characteristics [24] had studied that the live pathogenic *S. aureus* obtained from cervical cultures decreases in motility and viability of human spermatozoa which was absent in the sets in which the bacteria had been killed by boiling for 30 min before mixing the ejaculate. Another study by Hosseinzadeh *et. al.,* (2010) [25] reported that elementary bodies of *Chlamydia trachomatis* Serovar E can induce premature sperm death upon co-incubation with human sperm but this effect is abolished when these EBs have been previously killed by heat treatment.

Alternatively, effect of antimicrobial agents on bacteria was also observed. Treatment of cultured bacteria with different groups of antibiotics abolished the effect. This indicates that there must be some adhesion sites on spermatozoa to which *E. coli* binds.

The mechanisms by which *E. coli* affect sperm functions have not yet been identified. Interference of *E. coli* with these receptors may influence the motility and viability. Other authors reported similar observations concerning sperm motility and agglutination after incubation with *E. coli* [23; 16].

Thus, we suggest that *E.* coli/spermatozoa-interaction may be a two-step process: i.e. adhesion to and subsequent destruction of the sperm membrane. This mechanism may account for any inhibitory effects of *E. coli* infection on male fertility. It is speculative whether the same mechanisms are also responsible for impaired male fertility in cases of genitourinary infections.

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