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Agglutination of Human Spermatozoa Due to Human Semen Culture Bacterial Isolates Bearing Sperm Ligand

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Abstract

Sperm ligand on *Escherichia coli* that agglutinates human spermatozoa has been extracted from washed *E. coli* cells by incubating with 3M NaCl for 12 h at 37 ℃ under shake conditions (150 rpm). Further the sperm ligand was purified after extensive dialysis against double distilled water by step down chromatography procedures (molecular weight sieving, anion exchange). The purification procedure yielded a protein of apparent homogeneity. The ligand was heat labile as it could not withstand a temperature of 60 ℃ for 10 minutes. The ligand did not pass through the dialysis tubing. The molecular weight was found to be ~71KDa. Electron microscopy studies of washed sperm cells treated with purified ligand showed profound morphological alterations to head, neck and tail.

Keywords: *E. coli*; ligand; spermatozoa; agglutination; scanning electron microscopy

1.0 Introduction

For successful fertilization, motility is the most obvious and most essential sperm function and has been repeatedly shown to be predictive of fertilization in vitro (Bonde *et al*., 1998). Several studies have shown that the motility characteristics of spermatozoa are of the utmost importance for the men's fertility (Karpuz *et al*., 2007). Spermatozoa dysfunction is the single most important cause of infertility. A decrease in spermatozoa motility with time is universal phenomenon. This reduction differs from species to species and also among individuals of the same species, as in the human male. Most investigators agree that the majority of spermatozoa cease to move within the first 24 hours. The survival of spermatozoa after ejaculation is dependant on the environmental conditions under which they are kept. In the female genital tract they may remain

active for several days (Hafez, 1980), but their activity is of much shorter duration if they remain in the seminal fluid outside the body.

Male genital infections are relevant cause in the etiology of infertility due to abnormalities in sperm quality (Golshani *et al*., 2006; Pellati *et al*., 2008), affecting spermatozoal count and motility. The comparison of semen characteristics between infected and non-infected men show that motile spermatozoa and viability are lower when the microorganisms are present in the semen (Menkveld *et al*., 2001). It appears that bacteria have a direct effect on semen quality with negative consequences in fertility.

Men may have infections of their reproductive tract, these may include infection of the prostate (prostatitis), of the epididymus (epididymitis), or of the testis (orchitis). The principal microorganism causing prostatitis and epididymitis is *E. coli* (Giamarellou *et al*., 1984). The significance of asymptomatic infection or bacterial colonization of the male genital tract is less well-known and the interpretation of semen cultures in a fertility clinic is puzzling.

E. coli is one of the most frequent microorganism isolated from the ejaculate (Huerta *et al*., 2002). Several reports describe sperm agglutination and immobilization by *E. coli* (Huwe *et al*., 1998; Khalili & Sharifi-Yazdi, 2001). Paulson & Polakoski (1977) investigated the mechanism of how *E. coli* immobilizes spermatozoa and they reported a factor, apparently excreted by the bacteria which immobilizes spermatozoa without agglutinating it. However, Diemer *et al.* (1996) reported that *E. coli* inhibits sperm motility by directly adhering to and agglutinating spermatozoa. Rapidity and extent of sperm–*E. coli* agglutination indicated strong adhesive forces. Bartoov *et al.* (1991) proposed that mannose plays a critical role in adherence of *E. coli* to sperm. *E. coli* adherence is mediated by mannose residues present on the sperm. But what are the ligands? The purpose of this study is to investigate about the human sperm ligand on *E. coli* that binds to mannose present on the sperm.

2.0 Materials and Methods

Sperm Samples

Spermatozoa were obtained from healthy donors and men undergoing evaluation of fertility at PGIMER, Chandigarh, India, by masturbation following a 24 h continence period. Only ejaculates showing sperm parameters according to World Health Organization criteria (1999) were used. Depending upon the experiment, ejaculates were used unwashed or were washed twice with PBS (50mM, pH 7.2) and suspended in PBS. The number of sperm was checked by counting in a hemocytometer and was adjusted to 40 x 10^6 spermatozoa ml^{-1}.

Bacterial Samples

Semen samples of 10 males attending to infertility clinic (General Hospital, Sector-16, Chandigarh) were collected in the clinic by masturbation following a 24 h continence period. Before taking the semen samples the patient's recent medical history was taken into consideration. The semen samples were taken only from only those males who were without clinical symptoms of urogenital infections and who had not had antibiotic intake for atleast a week. All the patients were of the age group of 20-40 years. Samples were collected in sterile wide mouth plastic container. Samples underwent liquefaction at room temperature for 30 minutes. Then the samples were streaked on Blood agar plates separately and the plates were incubated aerobically at 37℃ for 24-48 h. The isolates were identified according to Bergey's Manual of Determinative Bacteriology (1994). The most abundant microorganism was *Staphylococcus* while *E. coli* could be isolated from only two males. Screening of both the *E. coli* isolates for interaction with human sperm identified both of them as an inducer of sperm agglutination. The isolate (No. 2) giving maximum agglutination was selected for further studies.

Sperm Bacteria Interaction

The isolate giving maximum agglutination (Isolate No. 2) was grown in Luria Broth (LB) under shake conditions (150 rpm) at 37℃ for 48 h. The culture was centrifuged at 10,000 g for 10 minutes at 4℃. Cell free supernatant was prepared by passing the supernatant through a 0.22μm

Millipore filter. The bacterial cells were washed twice with sterile PBS (50mM, pH 7.2). Equal volumes of semen samples (40 x 10^6 spermatozoa ml^{-1}) and bacterial cell culture (10^7 cells ml^{-1})/washed cells/cell free supernatant were mixed and incubated at 37℃ for 2 h. One drop of each was placed on a glass slide covered with a coverslip and observed for agglutination at 400X magnification under light microscope (Getner, Ambala, India). As a control, a sterile growth medium was used. As only cell culture and washed cells were able to agglutinate the spermatozoa, whereas culture supernatant failed to do so, therefore, further studies were carried out with washed cells. Pretreatment of *E. coli* by sonication produced bacterial fragments that were still able to agglutinate spermatozoa. The centrifugation of *E. coli* fragments at 10,000 g for 5minutes completely removed sperm-agglutinating elements from the solution.

Extraction of Sperm ligand

Extraction of sperm ligand from washed *E. coli* cells (48 h old) was done by salt washing method. The washed cells of *E. coli* (1000ml, 48 h old cell culture) were incubated with 1, 2, 3, 4 and 5M solution of NaCl under shake conditions (150 rpm) at 37℃ for different time intervals 2, 4, 8, 12 and 24 h, separately. Then the cells were centrifuged at 10,000 g for 30 minutes. The resulting cell pellet and supernatant (which was dialyzed against double distilled water overnight at 4℃ and passed through the UM05 Amicon filter) were analyzed for sperm ligand. As pellet did not show any spermatozoal agglutinating activity, further work was carried out with the supernatant.

The washed cells of *E. coli* were also subjected to sonication. 1000ml of 48 h old cell culture of *E. coli* was centrifuged at 8000 g for 15 min and the cells collected were sonicated (B.Braun, Labsonic 2000 Ltd.) for 10 min and then centrifuged at 10,000 g for 30-45 min. Both the cell debris and the sonicated supernatant were checked for agglutination activity. The sperm agglutinating activity was absent in sonicated supernatant. Further the cell debris was treated with different molarities of NaCl solution at 37℃ for different time intervals as done with washed cells. The solution was then centrifuged at 10,000 g and both cell debris and dialyzed supernatant were checked for the agglutination of spermatozoa. The agglutinating activity was absent in the cell debris so the further study was done with the dialyzed supernatant. Amongst the two methods of extraction described above, salt washing was found to be more efficient in extracting the sperm ligand from washed *E. coli* cells then sonicated cells (each prepared from 1000 ml of 48 h old cell culture). Therefore ,this was used as a method of choice for extraction of sperm ligand on *E. coli*. Boiling of supernatant for 10 min waived its spermatozoal agglutinating activity indicating that the active component may be a protein.

Purification of Sperm ligand

Purification of crude sperm ligand consists of filtration of dialyzed and concentrated fraction extracted with 3M NaCl (Qualigens Fine Chemicals, India) through a Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala) column. Fractions of 3ml each were collected and were read at 280nm UV spectrophotometer. Fractions showing agglutinating activity were pooled and concentrated using PEG 6000 under cold conditions.

The fractions concentrated after molecular sieving through G-200 were applied to DEAE cellulose (Hi-Media laboratories, Mumbai, India) column. Final elution was done with 0.05, 0.1, 0.2, 0.4 and 0.6M NaCl dissolved in PBS (50mM, pH 7.2). Fractions of 4ml each were collected and read at 280nm on UV spectrophotometer (Hitachi U-2900). Fractions showing agglutinating activity were pooled and concentrated. Polyacrylamide gel electrophoresis (PAGE) of purified sperm ligand was carried out to check the purification status. Molecular weight was estimated by sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) using the standard molecular weight markers.

Scanning Electron Microscopy (SEM)

SEM on a Jeol Scanning Microscope (JSM-6100, Jeol, Japan) was done to study the site of adherence of *E. coli* to spermatozoa and the effect of purified sperm ligand on spermatozoa.

Processing of samples was done according to the method described by Hafez & Kanagawa (1973) with slight modifications. For electron microscopy, sperm- *E. coli*/ sperm ligand agglutinates were mixed in 2.5% phosphate buffered glutaraldehyde and incubated at 37 ℃ for 30 minutes. The mixture was then washed thrice with PBS and suspended in 0.5ml of PBS. One drop of fixed and washed sample was placed on a silver–painted adhesive tape and mounted on brass stub and air dried. 100Å thick gold coating was done and the specimen was observed.

3.0 Results and Discussions

Bacterial infections have long been recognized as having an association with infertility. It is also known that bacteria are capable of agglutinating and immobilizing spermatozoa. Antibiotic treatment of spermicidal bacteria in women, men, and the ejaculate itself has resolved the infections and resulted in pregnancies in many of the previously infertile couples. Occasionally, some infertility problems have not been associated with frank infections but have presented as subclinical asymptomatic prostatitis in males whose initial sperm motility diminished over a several hour time interval.

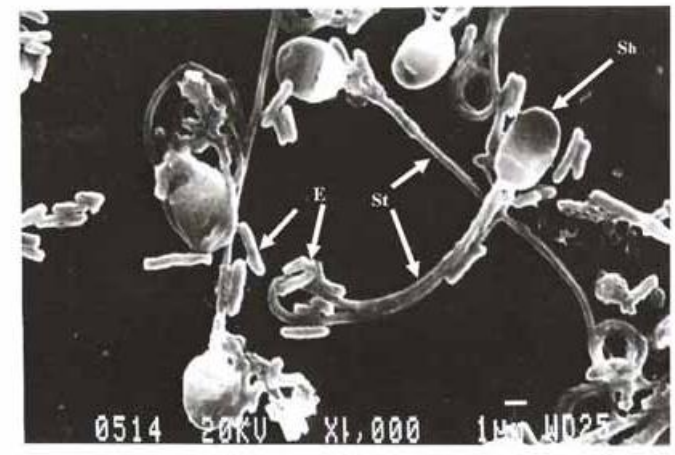

Figure 1. Scanning electron micrograph of *Escherichia coli* (E) adhered to both sperm heads (Sh) and tails (St) (at X 1000 magnification).

Bacteria affect sperm motility by adherence, agglutination and dialyzable factors. It is known that there is a significant negative effect of *E. coli, Enterococci* and coagulase negative *Staphylococci* towards sperm motility, morphology and viability (Esfandiari *et al*., 2002; Rodin *et al*., 2003, Sanack-Maciejewewska *et al*., 2005). Bacterial agglutination strong enough to induce hetero- agglutination of the microorganisms with motile sperm has been described for *E. coli.*

In the present study also *E. coli* isolated from asymptomatic males was shown to immobilize the spermatozoa. Sperm motility was inhibited by cell culture and washed cells but not by culture supernatant suggesting that agglutinating activity is associated with bacterial cells and not their metabolites.

Scanning electron microscopy showed close association between *E. coli* and sperm. Adherence of *E. coli* was seen on both the sperm head and tails (Fig.1). This observation is supported by the study made by Wolff *et al*. (1993) where they have reported the adherence of *E. coli* to both heads and tails of spermatozoa. However, *Staphylococcus aureus* was observed to adhere only to the tails of the spermatozoa and cause agglutination (Ohri & Prabha, 2005).

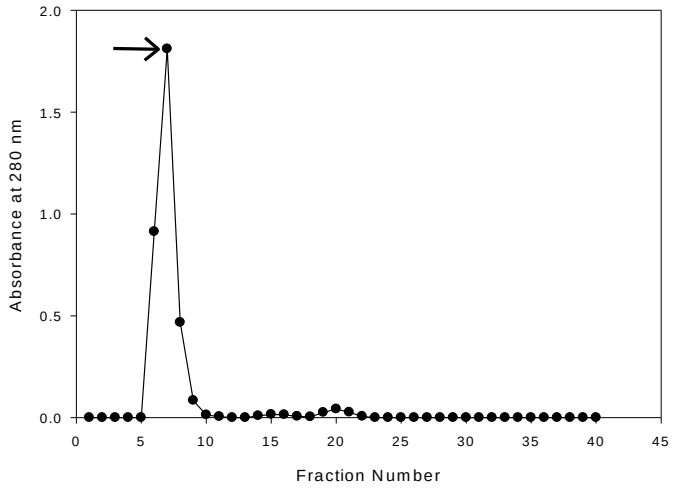

Figure 2. Column chromatographic pattern obtained after Sephadex G-200 gel filtration of dialyzed and filtered supernatant containing sperm ligand on *E. coli***,** showing the presence of ligand in fractions 6-9 with peak value in fraction 7 (arrow refers to sperm ligand on *E. coli*).

Pretreatment of *E. coli* by sonication produced bacterial fragments that were still able to agglutinate sperm. Centrifugation of *E. coli* fragments at 10,000 g for 5 minutes completely removed sperm agglutinating elements from the solution. Extraction of sonicated bacterial cells with 3M NaCl released the ligand into the solution. Sperm ligand could also be extracted from washed *E. coli* cells without sonication by 3M NaCl. This method gave better yield of sperm ligand as compared to sonicated cells.

Therefore, for further studies sperm ligand was extracted from washed *E. coli* cells without sonication. Sequential chromatography procedure included dialysis, molecular weight sieve chromatography and ion exchange chromatography on DEAE cellulose column.

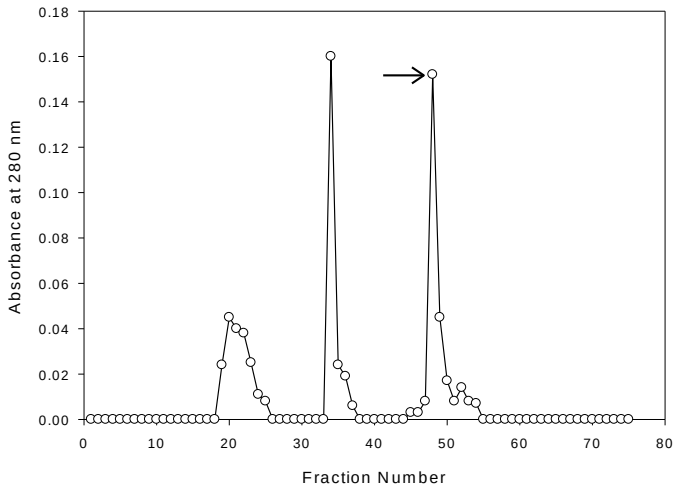

Figure 3. DEAE cellulose chromatography of G-200 pooled and PEG concentrated fractions revealed the presence of three peaks but the agglutinating activity was present only in the fractions 46-49 with peak value in fraction 48 (arrow refers to sperm ligand on *E. coli*).

The column chromatographic pattern on Sephadex G-200 (Fig.2) showed that the agglutinating activity was present in the fractions 6-9 with a peak value in fraction 7. The fractions showing agglutinating activity were pooled and concentrated using polyethylene glycol (PEG) and were applied to DEAE cellulose column. The sperm ligand could be eluted with PBS (50mM, pH 7.2) containing 0.4M NaCl (Fig.3). The fractions showing agglutinating activity were 46-49 with peak value in fraction 48. With this procedure the ligand was purified with a 42% recovery. Its molecular weight was estimated at ~71KDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 4). The ligand was found to be heat labile as it could not withstand a temperature of 60℃ for 10 minutes, which is in contrast to spermatozoal immobilization factor isolated from *E. coli* by Paulson and Polakoski (1977), which was excreted in the medium and stable to heating, freezing and lyophilization.

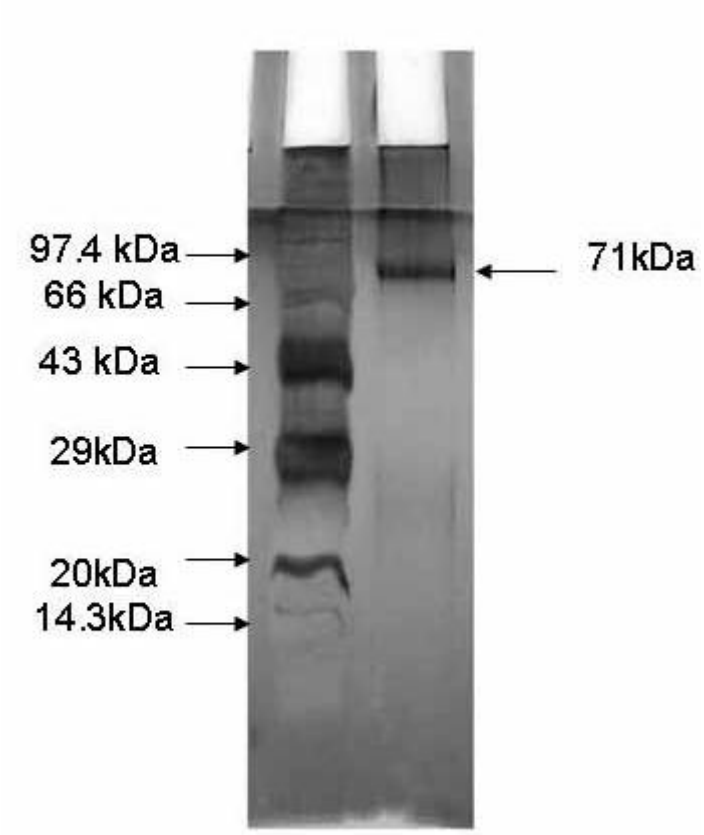

Figure 4. SDS-PAGE of purified sperm ligand, with **Lane1** containing Standard protein marker and **Lane2** containing DEAE cellulose purified and concentrated fraction. Molecular weight approx 71 kDa.

Minimum effective concentration of purified sperm ligand showing immediate agglutination of spermatozoa was 3.2 mg ml^{-1}. Electron microscopy studies of washed sperm cells treated with purified sperm ligand showed profound morphological alterations on human spermatozoa

including curling of tail (Fig.5), indicating that the morphological defects account for the immobilization of spermatozoa by the ligand. In a similar study Diemer *et al*. (2000) have reported the presence of multiple alterations on ultrastructure of spermatozoa upon artificial inoculation with *E. coli*, revealing the damage on sperm plasma membrane as well as the acrosome, thereby leading to immobilization.

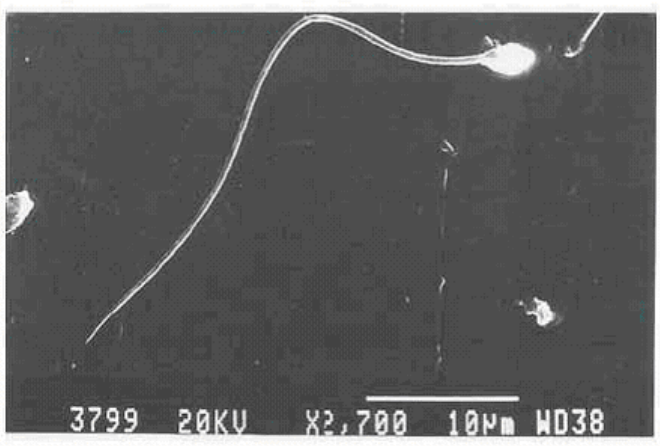

Figure 5a. Scanning electron micrograph of normal human spermatozoa (at X 2,700 magnification), serving as control.

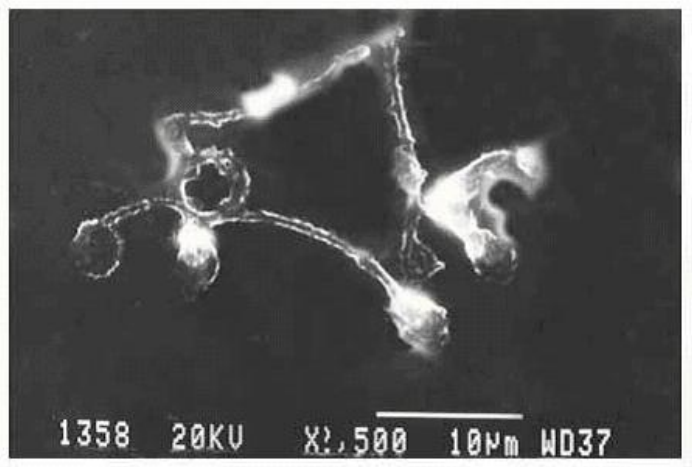

Figure 5b. Scanning electron micrograph of spermatozoa treated with purified sperm ligand (at X 2,500 magnification), showing the presence of morphological alterations on head, neck and curling of tail.

This study identifies a receptor- ligand interaction between *E. coli* and spermatozoa that results in sperm agglutination. Because the surface of spermatozoa is rich in glycoproteins, even asymptomatic colonization of male and female genitalia with bacteria may result in similar species specific interactions, causing agglutination of motile sperm.

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