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Research Article

Physicochemical Factors: Impact on Spermagglutination Induced by *Escherichia coli*

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Abstract. Motility is a sensitive parameter of sperm function which is predictive of its fertilization potential *in vitro*. The decrease in sperm motility may be associated with sperm agglutination and immobilization due to mere presence of bacteria or excretion of bacterial toxic products. Supplementation with various agents like sucrose, mannitol, calcium, and EDTA is well known to improve the sperm motility *in vitro*. The present study was designed to check any protective role exerted by the addition of different agents on spermatozoal motility against *E. coli* induced sperm agglutination. 52 semen specimens were screened for the presence of sperm-agglutinating strain of *E. coli*. Further, influence of various factors, namely, sugars, salts, and chelating agents was studied. Also, the impact of exposure to high temperature and alcohol on sperm-agglutinating efficiency of *E. coli* was observed. None of the factors could inhibit the sperm agglutination induced by *E. coli*, except high temperature suggesting the involvement of protein moiety. In addition, it was observed that agglutinating efficiency of *E. coli* was limited to spermatozoa and RBCs. It may be concluded that sperm-agglutinating property of *E. coli* is quite stable as various physicochemical factors tested did not show any negative effect on the same except high temperature.

Keywords: Escherichia Coli, Sperm Immotility, Agglutination, Semen Sample, Sugars, Salts

1. Introduction

Sperm is a highly specialized cell that must express diverse arrays of properties including motility, acrosome reaction, zona recognition, and fusion with oocyte [1]. Among all, sperm motility and viability are most essential for successful fertilization. Motility is an expression of the viability and structural integrity of the cell as the fertile life of a spermatozoon can be measured by the duration of its motility [2]. Hence, spermatozoa dysfunction is the single most important cause of infertility. The negative impact of some microorganisms relevant to genital infections on sperm function has been claimed [3]. Some possible pathomechanisms of the development of infertility linked with infection are considered: direct effect on sperm function (motility, morphology, etc.), deterioration of spermatogenesis, autoimmune processes induced by inflammation, and dysfunction of accessory sex glands [4]. Recent studies have shown that the simple presence of bacteria in semen samples may compromise the semen quality [5]. The above-said facts were further justified by an observation made by a group of scientists wherein improvement of semen quality following eradication of infection was seen [6].

Various microorganisms isolated from semen sample include *Escherichia coli, Enterococcus faecalis, Micrococci,*

Streptococcus agalactiae, Staphylococcus aureus, and *Morganella morganii*. Amongst all, *E. coli* represents the most frequently isolated microorganism in male patients with genital tract infections or semen contamination [5]. The negative influence of this species on sperm quality is partially due to its effect on sperm motility via sperm immobilization/agglutination. Paulson and Polakoski [7] 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. [7] reported that *E. coli* inhibits sperm motility by directly adhering to and agglutinating spermatozoa.

There is evidence that fertilization related events can be accomplished *in vitro* by incubating sperm under specific conditions [9]. Enhancement of sperm motility was seen with supplementation of spermatozoa using different sugars, salts, and chelating agents *in vitro*. Thus, the present study was aimed at the isolation of sperm agglutinating strain of *E. coli* from semen sample and determination of the influence of various factors, namely, sugar, salt, and chelating agents on sperm agglutination induced by bacteria. Further, effect of exposure of *E. coli* to temperature and alcohol on its sperm agglutinating efficiency was checked. In addition, agglutinating property of the isolate on other eukaryotic cells, namely, RBCs, spores of fungus spores ,and yeast cells was explored.

2. Material and Methods

2.1. Semen samples. Spermatozoa were obtained from males undergoing evaluation of fertility at Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, by masturbation following a 48 h abstinence period. Only ejaculates showing normal sperm parameters according to World Health Organization criteria [10] were used. The number of sperm was checked by counting in a haemocytometer and count was adjusted to 40×10^6 spermatozoa/ml using phosphate buffered saline (PBS; 50 mM, pH 7.2). The protocols for the present study were approved by the Panjab University Institutional Ethics Committee: vide letter no. 02/PUIEC dated 25.03.09

2.2. Bacterial Samples. Semen samples of 52 men undergoing semen analysis (PGIMER, Department of Urology, Chandigarh, India) were collected in the sterile wide mouth container by masturbation. Before taking samples, the patients' recent medical history was taken into consideration. The semen samples were taken only from those males who were without clinical symptoms of urogenital infections and who had not had antibiotic intake for at least a week. All the samples were rapidly transferred to laboratory and samples underwent liquefaction at room temperature for 30 minutes. Then, the samples were streaked on brain heart infusion (BHI) agar plates separately and the plates were incubated aerobically at 37 ˚C for 24–48 h.

Identification of the isolates showing the presence of Gram negative rods was carried out using Bergeys Manual of Bacteriology [11] and HiMotility Biochemical kit (HiMedia laboratories, Mumbai, India). In the present study, 7 isolates of *E. coli* were obtained.

2.3. Sperm Bacteria Interaction. All the *E. coli* isolates were grown in Luria broth (LB) for 48 h at 37 ˚C under shaking conditions (150 revolutions per minute (rpm)). 0.1ml of semen sample containing 40×10^6 spermatozoa/ml was mixed with 0.1ml of bacterial cultures and incubated at 37 ˚C for different time intervals viz. 30 min, 1, 2, 3, and 4 h. 10 μl of the mixture taken after each time interval was placed on a clean glass slide, covered with a coverslip and observed under X400 objective for sperm agglutination using a bright field microscope (Olympus India Pvt. Ltd.). As control, sterile uninoculated LB broth was mixed with semen sample and observed for sperm agglutination. On the basis of microscopic examination, a strain of *E. coli* giving maximum sperm agglutination was selected for further study.

2.4. Serotyping and Transmission Electron Microscopy. Serotyping of *E. coli* strain exhibiting maximum sperm agglutination was outsourced from Central Research Institute (CRI), Kasauli. Sample was prepared by stabbing 24 h old culture of the isolate in 2 ml of nutrient agar (NA) and sealed using parafilm under aseptic conditions.

Transmission electron microscopy (TEM) was carried out to look for the presence of fimbriae. A 2–3 mm colony of *E. coli* was inoculated in 10 ml of LB and allowed to grow for 48 h at 37 ˚C under shaking conditions. The cell culture was centrifuged at 10,000 rpm for 20 min and pellet so obtained was suspended in 100 μl of PBS. For transmission electron microscopy, *E. coli* cells were fixed in 6% glutaraldehyde and embedded in Epon Araldite. Postfixation was done by using 1% osmium tetraoxide in 100 mM phosphate buffer. The fields were observed for the presence or absence of fimbriae after mounting on copper grids.

2.5. Various factors influencing the agglutination reaction. The effect of various factors, namely, sugars, salts, chelating agents, temperature, and alcohol was checked on sperm-agglutinating efficiency of *E. coli*. For these experiments, aliquots of 2 ml of 48 h old culture grown in LB at 37 ˚C under shaking conditions (150 rpm) were used. All the aliquots were centrifuged at 10,000 rpm for 10 min and pellet so obtained was washed twice with PBS and suspended in the same buffer. 2 ml of bacterial suspension was used as such in case of temperature and alcohol treatment. Each experiment was carried out thrice with samples from different donors.

2.6. Sugars. 48 h old culture of bacteria was grown and divided into aliquots of 2 ml. Each aliquot was centrifuged and the pellet so obtained was washed twice with PBS. To

each aliquot, 0.1 ml of semen sample (40×10^6 spermatozoa/ml) was added. To determine the effect of various sugars, namely, sucrose, galactose, mannitol, mannose, maltose, dextrose, and xylose on sperm agglutination, different concentrations of sugar solutions were added in each aliquot in order to achieve a final concentration of 1 mM and 5 mM. As control, 0.1 ml of semen sample (40×10^6 spermatozoa/ml) was mixed with 0.1 ml of PBS or bacterial suspension or sugar solutions. On completion of incubation period, that is, after 2 h, one drop (10 μl) of each was placed on glass slide covered with a coverslip and checked for agglutination at X400 magnification under ordinary light microscope.

2.6.1. Salts. The effect of various salts, namely, $MnSO_4$, $MgSO_4.7H_2O$, NaCl, $(NH_4)_2SO_4$, KCl, $CuSO_4$, Na_2CO_3, and $NaHCO_3$ was examined on the sperm-agglutinating property of *E. coli*. For this, different aliquots containing 0.1 ml bacterial suspension and 0.1 ml of semen sample (40×10^6 spermatozoa/ml) were used. Different concentrations of salt solutions were added in each aliquot in order to achieve a final concentration of 1 mM and 5 mM, respectively. As control, 0.1 ml of semen sample (40×10^6 spermatozoa/ml) was mixed with 0.1 ml of PBS/bacterial suspension/salt solutions. After 2 h of incubation, agglutination induced by *E. coli* was checked by light microscopy which was compared with control.

2.6.2. Chelating agents. The effect of two chelating agents, namely, EDTA and sodium citrate, was checked on sperm agglutination by *E. coli*. For this, 0.1 ml of washed bacterial culture was mixed with 0.1ml of semen sample (40×10^6 spermatozoa/ml) and incubated with respective concentrations (1 mM and 5 mM) of EDTA and sodium citrate. As control, 0.1 ml of semen sample (40×10^6 spermatozoa/ml) was mixed with 0.1 ml of PBS or bacterial suspension or chelating agents. After incubation of 2 h, each sample was observed for sperm agglutination under light microscope.

2.6.3. Temperature. In order to study the effect of dead bacteria on sperm agglutination, the bacteria were killed using heat treatment. Aliquots of 2 ml of 48 h old culture of *E. coli* were exposed to different temperatures, namely, 40, 50, 60, 80, and 100 °C for 10 and 30 min and checked for nonviability by subsequent culturing on NA plates. To find out the effect of live and dead bacteria on sperm agglutination, 0.1 ml of fresh ejaculate (40×10^6 spermatozoa/ml) was mixed with 0.1 ml of the bacterial suspension on a glass slide and examined at X400 under the microscope. The stability of cell suspension was also checked at lower temperature, that is, 4 °C. For this, aliquots of bacterial suspension were kept in refrigerator for 4 weeks and sperm agglutination was checked by taking out 0.1 ml of suspension per week and examined as described above.

2.6.4. Alcohol treatment. Aliquots of 2 ml of 48 h old culture of *E. coli* were exposed to 10 volumes of 1, 10, 25, and 50% alcohol and kept at room temperature under shaking conditions (150 rpm) for 1 h. After incubation, all the aliquots were centrifuged at 10,000 rpm for 10 min and the pellet so obtained was washed thrice with PBS and suspended in the same buffer. For checking agglutination, equal volumes (0.1 ml) of these suspensions were mixed with semen sample (40×10^6 spermatozoa/ml) for 2 h. After 2 h, 10 μl from each aliquot was placed on glass slide and covered with coverslip to be examined under microscope at X400 magnification.

2.7. Effect of E. coli on other eukaryotic cells.

2.7.1. RBCs. 2% RBC suspension of human blood and 24 h old cell culture of *E. coli* was prepared, separately. 50 μl of PBS (pH 8.0, 50 mM) was added to each well of microtiter plate. To the first well, 50 μl of the bacterial suspension was added and serially diluted with PBS. Further, 50 μl of 2% suspension of human RBCs was added in each well. The plate was kept on a Microshaker and incubated at 37 °C for 2 h. The wells were checked with naked eye for button or mat formation at the bottom of each well.

2.7.2. Yeast cells and fungal spores. 24 h old culture of yeast (*Candida sp.*) and spores of one-month-old cultures of *Aspergillus niger* grown on a slanted Sabouraud's agar were suspended in 2 ml of saline. 0.1 ml of these suspensions was mixed with 0.1 ml of 24 h old cell culture of *E. coli* separately and incubated for 4 h. After each time interval, the slides were checked for agglutination microscopically.

3. Results

3.1. Characterization of the isolate. An ideal identification of isolate causing maximum sperm agglutination within 2 h of incubation was carried out using HiMotility biochemical identification kit (HiMedia Laboratories, Mumbai, India). Biochemical profiling and serotyping confirmed the isolate to be *E. coli* belonging to Rough type. TEM analysis revealed the strain to be fimbriated (Figure 1).

3.2. Factors affecting the agglutination reaction induced by E. coli.

3.2.1. Sugars. Coincubation of semen sample with different sugar solutions did not have any adverse effect on sperm motility except dextrose. Presence of dextrose itself had some inhibitory effect on sperm motility leading to 25% immotility in comparison to control. However, degree of sperm agglutination induced by *E. coli* was not inhibited in presence of any of sugar solution. It was observed that, within 2 h of incubation, *E. coli* resulted in 100% sperm agglutination except in case of dextrose wherein only 70%

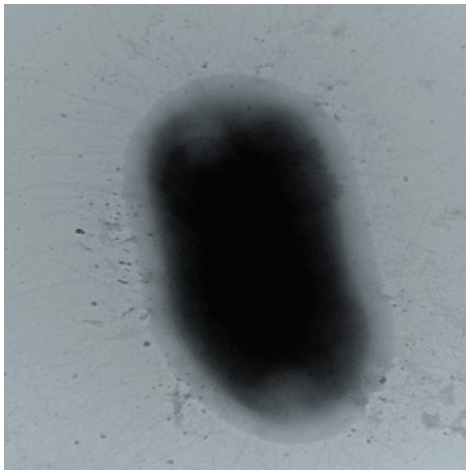


Figure 1: Transmission electron micrograph of *E. coli* showing presence of fimbriae.

of agglutination of motile cells was seen. These results suggested that none of the sugar had protective effect on sperm against the agglutination induced by *E. coli.*

3.2.2. Salts. Effect of various salts on sperm-agglutinating property of *E. coli* was seen. Results showed that $MgSO_4.7H_2O$ and $CuSO_4$ had negative effects themselves on the motility of spermatozoa at both the concentrations of 1 mM and 5 mM though no effect on efficiency of *E. coli* induced sperm agglutination was found in the presence of any of the salts.

3.2.3. Chelating agents. Effect of chelators like sodium citrate and EDTA was observed on sperm-agglutinating efficiency of *E. coli*. Results showed that presence of sodium citrate and EDTA did not inhibit immobilization of spermatozoa induced by *E. coli*.

3.2.4. Temperature. In order to bring about killing of bacteria, aliquots of 48 h old culture were exposed to different temperatures, namely, 40, 50, 60, 80, and 100 °C and plated on NA to obtain viable count. It was observed that exposure to 80 °C and 100 °C for 10 min could bring about complete killing of bacteria. In case of lower temperatures, about 4- and 3- log decrease in cell count was observed at 60 °C and 50 °C for 30 min, respectively, while no decrease was found in case of 40 °C. After heat treatment, incubation of live and dead bacteria with spermatozoa was carried out to study the effect of live and dead bacteria on sperm agglutination. For this, 0.1 ml of bacterial suspensions killed by heat treatment was incubated with 0.1 ml semen sample, separately. Agglutination was checked at different time intervals. As positive control, live bacteria were incubated with semen.

Mixing of live bacteria with semen leads to agglutination and dampening of sperm motility which increased with time. No agglutination was observed even after 2 h of incubation with bacterial suspension exposed to 80 °C and 100 °C (Figure 2). Hence, it was evident that only live *E. coli* can significantly depress the motility of human spermatozoa via agglutination and indicated that active component involved in sperm agglutination might be protein in nature. Also, no negative impact on sperm-agglutinating efficiency of *E. coli* was observed even after 4 weeks of exposure at 4 °C as 100% sperm agglutination could be seen.

3.2.5. Alcohol. Effect of different concentrations of alcohol was seen on sperm-agglutinating potency of *E. coli.* It was observed that even after the treatment of culture with 10 volumes of 1, 10, 25, and 50% ethanol for 1 h, no adverse effect on sperm agglutination was seen (Figure 3).

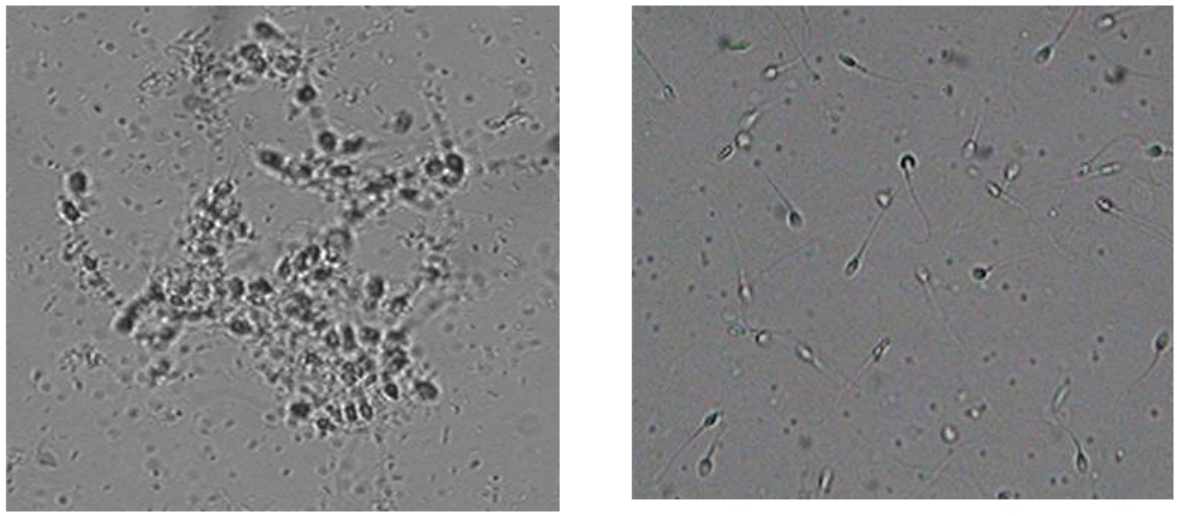
3.3. Effect of E. coli on other eukaryotic cells.

3.3.1. RBCs. Results showed the presence of haemagglutination activity of *E. coli* towards human RBCs with titer of 1:16 as evident by agglutination of RBCs with culture while button formation at the bottom of the wells was due to unlysed RBCs in control.

3.3.2. Yeast and Fungal spores. Incubation of *E. coli* with either yeast or fungal spores could not induce agglutination in any case even after incubation till 4 h.

4. Discussion

The negative impact of some microorganisms relevant to genital infections on sperm function has been claimed. Changes in sperm parameters that could account for infertility include reduced cell counts, reduced motility, or morphological alteration [12]. It is already known that these parameters play a vital role in the fertility potential of a man. Moreover, if spermatozoa prematurely lose motility, they also lose their natural fertilization potential since they cannot travel to meet oocyte. Among various microorganisms associated, *E. coli* appears to be the most important pathogen isolated from the ejaculate [13]. The direct inhibitory effect of *E. coli* on progressive motility of spermatozoa is being reported. Inhibition of motility is either directly by agglutination or indirectly by the secreted products leading to immobilization. This study presents agglutination of spermatozoa by *E. coli* obtained from the ejaculate of males attending infertility clinic. Serotyping of selected *E. coli* strain revealed it to be of rough type. The strain was capable of causing 100% sperm agglutination within 2 h of incubation. The agglutination was of mixed type, that is, head to head, head to tail, and tail to tail. These kinds of direct interactions between bacteria and spermatozoa have already been discovered for



(a) (b)

Figure 2: (a) Agglutination of motile spermatozoa by live *E. coli* (X 400) and (b) no agglutination by dead *E. coli* (X 400).

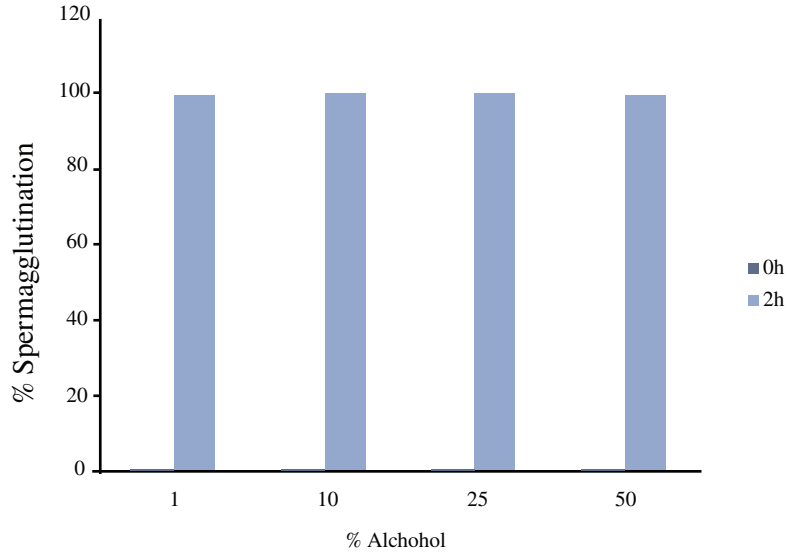


Figure 3: Effect of alcohol treatment on sperm agglutination by *E. coli.*

different bacterial species such as *Mycoplasmas*, *Ureaplasma urealyticum* [14], and *Chlamydia* species [15].

Though various studies are available in the literature in regard to the sperm-agglutinating property of *E. coli*, very few have checked the impact of important factors such as temperature and alcohol on *E. coli*. Teague and Nelson [16] reported complete absence of sperm agglutination on incubation with heat killed *E. coli*. Similarly, in the present study exposure of bacterial suspension at 60 °C for 10 min decreased the percent agglutination to mere 30% while no agglutination was observed at higher temperatures. The absence of immobilization and agglutination of spermatozoa upon incubation with killed *E. coli* could be suggestive of some labile factor present on bacterial cells which is taking part in agglutination. However, this mechanism clearly requires further investigation. Hosseinzadeh et al. [17] have also shown that live Chlamydial elementary bodies (EBs) can have a direct and detrimental effect on sperm physiology whereas heat treatment abolishes the same. Similar results were also obtained in case of *S. aureus* [18].

Further to examine any adverse effect applied by alcohol on agglutinating property of *E. coli*, aliquots of bacteria were exposed to different percentages of alcohol. No decrease was seen in agglutinating efficiency of *E. coli* even after the

exposure to 50% of alcohol. These results are in accordance with earlier studies made by Rosenthal [19]. It was reported that cells of *E. coli* mixed with 10 volumes of neat alcohol still had the capacity to agglutinate spermatozoa efficiently.

Reports from earlier studies showed increased total active sperm motility on supplementation with different sugars, namely, xylose, sucrose, and mannitol [20–22]. Stimulation of progressive motility of human washed sperm was reported in presence of manganese (Mn^{2+}) and potassium (K^{+}) at particular concentrations [23, 24]. Impact of different chelating agents has also been reported. Data showed decreased motility in presence of sodium citrate while improved semen motility was seen in case of EDTA [25]. The finding that supplementation with various agents such as sugars, salts, and chelating agents improves the motility of spermatozoa *in vitro* prompted us to carry out few experiments. The study was designed to check whether the addition of any of these agents can enhance the motility of spermatozoa in presence of *E. coli*.

When the effect of various sugars was examined, results showed that the addition of none of the sugar could inhibit the sperm agglutination induced by *E. coli*. These results are in contrast to the earlier studies wherein the addition of mannose, gal-gal, or α-methyl mannopyranoside blocked sperm-*E. coli* agglutination [26, 27]. Bartoov et al. [28] proposed that mannose plays a critical role in adherence of *E. coli* to sperm and inhibition of this interaction by mannose is indicative of involvement of type-1 fimbriae. However, from the results of the present study it may be suggested that adherence of isolated strain of *E. coli* does not involve type-1 fimbriae.

Coincubation of different salts with *E. coli* and sperm was carried out to assess any protective role exerted by them. The agglutinating reaction of *E. coli* was not inhibited in the presence of any of the salts. However incubation of two salts $MgSO_4$ or $CuSO_4$ with sperm alone had negative impact on sperm motility. Adverse effect on motility by Cu^{2+} and Mg^{2+} has already been reported at similar concentrations; hence, the results of the present study are in line with previous reports [24, 29]. No blocking effect was seen in case of the chelating agents as the addition of neither sodium citrate nor EDTA blocks the agglutination reaction.

So far very few studies have correlated the manifestations of an agglutinating factor inherent in strains of *E. coli*. Also, references in this subject are very few and concern only agglutination of red cells and spermatozoa. Therefore, to determine how widely the agglutinating property is encountered with this rough *E. coli*, it was incubated with RBCs, spores of fungus and yeast cells. The strain could induce agglutination of RBCs while no agglutination was found in the remaining two. These results are in accordance with earlier study made by Rosenthal [19] wherein few *E. coli* strains capable of causing agglutination of RBCs had no effect on other cells.

5. Conclusion

Bacteriospermia has a negative effect on sperm quality. Amongst all, *E. coli* is the most common bacteria with negative influence on sperm motility. Various factors including sugars, salts, alcohol, and chelating agents did not show any adverse impact on sperm-agglutinating efficiency of *E. coli*.

Conflict of Interests

The authors declare that they have no conflict of interests.

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