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Impact of Uropathogenic Microorganisms on Mouse Spermatozoa: an *In Vitro* Study

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Abstract

The effect of bacteriospermia on sperm quality is in the field of debate due to scarcity of related pertinent information hence, the present study attempts to elucidate the impact of various microorganisms found in male urogenital tract on mouse sperm parameters. In vitro impact of seven uropathogens on mouse sperm parameters viz. motility, viability, morphology, Mg^{2+}dependent ATPase activity and acrosome reaction was evaluated. The results revealed that out of seven uropathogenic microorganisms, five strains i.e. S. marcescens, E. aerogenes, K. pneumoniae, E. coli and S. aureus could negatively alter sperm parameters and cause sperm immobilization via agglutination. Scanning electron microscopy revealed that these strains could successfully adhere to spermatozoa and led to numerous morphological alterations. These strains significantly reduced the Mg^{2+}dependent ATPase activity and caused premature acrosomal loss. These uropathogenic strains were also able to cause 100% sperm death. Whereas, remaining two strains i.e. P. mirabilis and S. pyogenes did not result in any negative influence on sperm parameters viz. motility, viability, morphology, Mg^{2+}dependent ATPase activity and acrosome reaction. Hence, this study demonstrates that the presence of these uropathogens in male genitourinary tract or as a contamination in semen can significantly affect the sperm parameters and hence, negatively affect the fertility status.

Keywords: *Uropathogens, Spermatozoa, agglutination, scanning electron microscopy, Mg^{2+}ATPase, acrosome reaction.*

Abbreviations/ Acronyms

DMSO Dimethyl sulfoxide; SA Spermagglutinating; NSA Non spermagglutinating; PSA *Pisum sativum* agglutinin; FITC Fluorescein isothiocyanate; ATP Adenosine triphosphate.

Introduction

Infertility is a global health issue affecting 15% of all couples worldwide (Elbhar, 2005). Though socially less acknowledged, still males contribute about 40-50% of all infertility cases worldwide (Brugh and Lipshultz, 2004). Male infertility can be attributed to multiple factors which cause the sperm dysfunction, in terms of alterations in sperm concentration, motility, viability and morphology. The major causes of male infertility include congenital or acquired urogenital abnormalities, genitourinary tract infections, increased scrotal temperature (e.g. as a consequence of varicocele), endocrine disturbances, genetic abnormalities and immunological factors (Jungwirth *et al*. 2012). Out of all these factors, genitourinary tract infections play an obstructive and hidden role in male infertility. These infections, caused by various infectious agents such as bacteria, fungi, viruses and parasites, may impair important reproductive functions in males (Elbhar, 2005). The microorganisms responsible for semen contamination generally originate from the urinary tract of patients or can be transmitted by the partner during sexual intercourse (Purvis and Christiansen 1993). The negative impact of sexually transmitted microorganisms on spermatozoa has been observed (Tian *et al*. 2007). Since, they can easily adhere to spermatozoa, leading to the morphological changes/structural alterations

which result in decrease in the fertilization potential of sperm (Comar *et al*. 2013). But till date the role of uropathogenic microorganisms on sperm parameters is being debated. Hence, the present study sheds the light on the impact of some common uropathogenic microorganisms on sperm parameters.

Materials And Methods

Animals

Sexually mature male BALB/c mice (5-6 weeks old, 25±2g) were procured form the Central animal house of Department of Microbiology, Panjab University, Chandigarh, India. The animals were given standard pellet food with water *ad libitum* and maintained (12:12, dark:light cycle) under standard laboratory conditions. All the experimental procedures were approved by Institutional Animal Ethics Committee, Panjab University vide letter no. PU/IAEC/S/15/67 dated 15.09.2015. All experiments were successfully completed in agreement with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Microorganisms

A strain of *Escherichia coli* (previously isolated in our laboratory from semen of males undergoing analysis at PGIMER, Chandigarh), *Staphylococcus aureus* (previously isolated from the cervix of a woman suffering from unexplained infertility, attending the Department of Obstetrics and Gynecology, Government Multi Speciality Hospital, Sector-16, Chandigarh, India) and the five standard uropathogenic strains of *Serratia marcescens* (MTCC 7641), *Enterobacter aerogenes* (MTCC 7324), *Klebsiella pneumoniae* (MTCC 4030), *Streptococcus pyogenes* (MTCC 1924) and *Proteus mirabilis* (MTCC 425) were procured from the Microbial Type Culture Collection, Institute of Microbial Technology, Sector 39, Chandigarh, India. The strains were grown in Brain heart infusion broth and maintained as glycerol stocks at -80°C. A fresh stock was used for each experiment.

Extraction of Spermatozoa from Mice

The 6-7 week-old BALB/c male mice were sacrificed by cervical dislocation, and spermatozoa from the vas deferens were collected in prewarmed 50mM PBS by gentle teasing and the count was set at 40 × 10^6 spermatozoa/ml, unless stated otherwise.

In Vitro Impact of Various Uropathogens on Mouse Spermatozoa

Effect of Cell Culture, Cell Free Supernatant and Washed Cells of Uropathogenic Microorganisms on Motility and Viability of Spermatozoa

All the five standard uropathogens along with two bacterial isolates were grown separately in brain heart infusion (BHI) broth under shaking conditions (150 rpm) at 37ºC. In each case, 0.2 ml inoculum of overnight culture of the each microrganism was used to inoculate 20 ml of growth medium (BHI). The flasks were incubated for 24, 48 and 72h at 37ºC. The cultures were centrifuged at 10,000 rpm for 20 min at 4ºC and clear supernatant was separated. The cell pellet obtained was washed twice with PBS (50 mM, pH 7.2) and resuspended in the same buffer. To check the effect on motility and viability, 100 µl of mouse sperm suspension was mixed with 100 µl of cell culture/ washed cells/culture supernatant and incubated for 30 min at 37 ºC. After different time intervals, a wet preparation using 10 µl aliquot of the mixture was observed at 400X magnification using a bright-field microscope. A control containing BHI broth mixed with a sperm sample was set up simultaneously. Minimum ten fields were scanned and mean number of spermatozoa per field along with motility (%) were calculated. In order to assess the percentage of viable sperms, an equal volume of sperm sample was mixed with cell culture/washed cells/ culture supernatant and 0.5% eosin and examined under the light microscope at 400X magnification. Any degree of pink/red stained spermatozoa was counted as 'dead' while unstained (white) as 'alive'. From the results, it was observed that only washed cells could affect motility and viability hence, for further experiments washed cells were used.

Morphology

The effect of uropathogenic microorganisms on morphology of spermatozoa was assessed using scanning electron microscopy (SEM) at Sophisticated Analytical Instrumentation Facility (SAIF), Panjab University, Chandigarh. The samples were processed in accordance with the standard method described by Hafez and Kanagawa (1973). Mouse spermatozoa were centrifuged at 1000 rpm for 10 min. The supernatant was discarded, the pellet was washed twice with PBS and resuspended in same PBS buffer to get a washed sperm suspension containing 40

×10^6 spermatozoa/ml. 200µl of this suspension was mixed with equal volume of washed cells of different uropathogenic microorganisms and incubated for 2h. After incubation, spermatozoa were fixed with 4ml of 2.5% phosphate buffered gluteraldehyde at 37ºC for 30min. Fixation was followed by washing of samples with PBS and finally suspending the same in distilled water. A drop of suspension was placed on a silver painted adhesive tape mounted on brass stubs and air dried. 100Å gold coating was done using fine coat, Jeol ion sputter (JFC-1100). This gold coated stub was analysed at different magnifications under the Scanning Electron Microscope (model JSM-6100, SM-Jeol 20kV).

Mg^{2+}Dependent ATPase Activity

Mouse spermatozoa were centrifuged at 500 rpm for 10 min and the sperm pellet was suspended in Tris-HCl (0.2M, pH 7.6). Washed spermatozoa (1× 10^6/ml) were sonicated at 50Hz for 10min (10 cycles of 30s with 1min interval) at 4ºC. The reaction mixture for ATPase consisted each of Tris-HCl buffer (0.2M, pH 7.6), $MgCl_2$ (5mM), ATP (6mg/ml) and sonicated sperm suspension. The washed cells of sperm agglutinating (SA) or non sperm agglutinating (NSA) microorganisms were added to the reaction mixture and incubated at 37ºC for 1h. The reaction was stopped by adding 1ml of 10% cold Trichloroacteic acid (TCA) and then incubated at 4ºC overnight for protein precipitation. The control tubes contained all the components of the reaction mixture but TCA was added immediately after mixing the components. Inorganic phosphate (Pi) released after breakdown of ATP was determined according to the method of Boyce *et al*. (2004). Mg^{2+}ATPase activity of spermatozoa was studied according to Kielley (1955) and Chappel (1961). The ATPase activity was estimated as the amount of enzymatically released inorganic phosphate (Pi). One unit of ATPase was expressed as the micromoles of Pi released after 1 h of incubation at 37 °C.

Evaluation of Acrosomal Status of Spermatozoa

The washed mouse spermatozoa upon resuspension in Ham's F-10 medium (containing HEPES and 1% human serum albumin) was incubated for 3 h at 37 °C, post which spermatozoa were collected by centrifugation (500 × g for 10 min) and their motility was assessed. The aliquots of motile spermatozoa (20× 10^6 cells) incubated with either of 0.1% DMSO (-ve control) or 10 µM Calcium ionophore A23187 (+ve control) or 0.1 ml of washed cells and incubated for 1 h at 37 ºC. For the assessment of acrosomal status, a smear of sperm pellet was prepared on a glass slide, fixed in 95% ethanol for 30 min, air dried, washed in distilled water for 10 min and stained for 4 h with 25 µg/mL PSA-FITC in PBS (pH 7.4) at 4 °C. The *Pisum sativum* agglutinin-Fluorescein isothiocyanate (PSA-FITC) solution was washed with distilled water, air dried and covered by 30 µL of anti-fading medium (50% v/v glycerol, 50% v/v distilled water, 25 mg/mL 1′, 4-diazabicyclo [2, 2, 2] octane) and at least 100 spermatozoa were counted using a fluorescence microscope at 1000X. The scoring was done as 'acrosome intact' or 'acrosome reacted' depending on the presence or absence of uniform specific fluorescence restricted to the acrosome cap respectively (Cross and Hanks, 1991).

Results

Effect of Cell Culture, Cell Free Supernatant and Washed Cells on Motility/Viability of Spermatozoa

In order to assess the effect of these uropathogens on sperm motility, 24, 48 and 72h old respective bacterial cultures were incubated with mouse spermatozoa. The results showed that *S. marcescens*, *E. aerogenes*, *K. pneumoniae*, *E. coli* and *S. aureus* could negatively alter sperm parameters as compared to control sample. They were found to exert a significant atrocious effect on sperm motility by causing agglutination. Out of the sperm agglutinating cultures grown for different time intervals, 72h old cultures were found to be most efficient in causing complete agglutination within 45 min **(Figure 1)**. On the other hand, incubation of spermatozoa with 24/48/72h old cultures of *S. pyogenes*/*P. mirabilis* did not result in any negative influence on sperm motility at any of the specified time intervals **(Figure 2)**.

Further, to evaluate if the phenomenon of sperm agglutination is associated with surface bound factors or extracellular factors, mouse spermatozoa were incubated with washed cells or cell free supernatants of sperm agglutinating 72h old microbial cultures **(Figure 1a)**. The results indicated that only washed cells of all the sperm agglutinating bacteria could effectively agglutinate spermatozoa, while their cell free supernatants failed to do so. In case of *S. marcescens,* the occurrence of agglutination became visible within 15min and after 30 min of incubation 100% agglutination could be observed. Whereas, upon incubation of mouse spermatozoa with washed

cells of *E. aerogenes*/*K. pneumoniae*/*E. coli*/*S. aureus* complete agglutination could be observed at 45 min of incubation **(Figure 1a, 1b).**

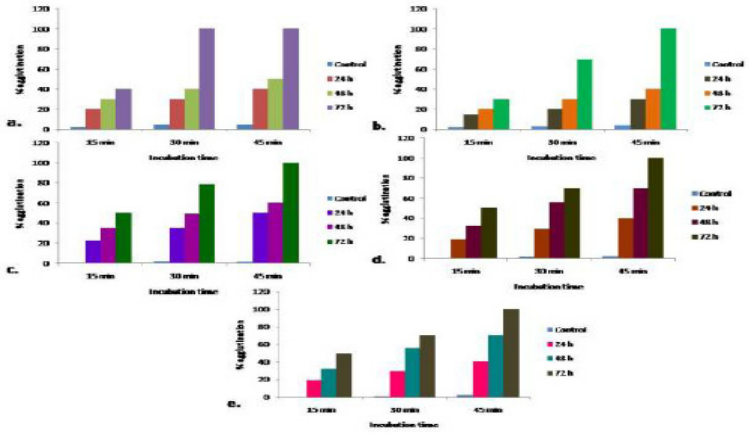


Fig 1a. *Figure illustrating the percentage agglutination at different time intervals after coincubation with 24/48/72h old culture of S. marcescens (a), E. aerogenes (b), K. pneumoniae (c), E. coli (d) and S. aureus (e)*

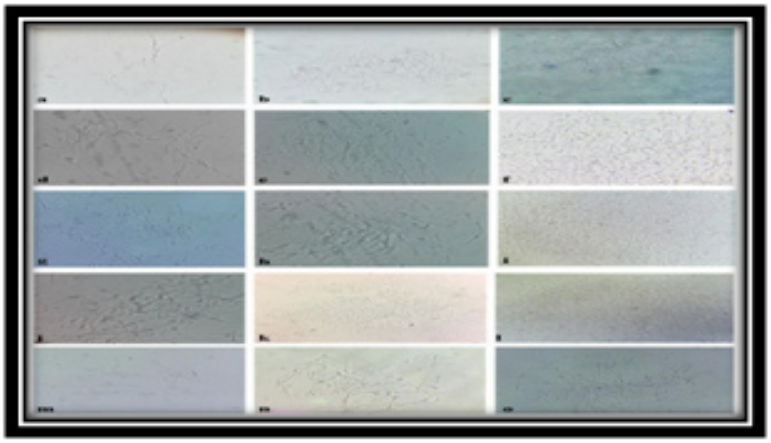


Fig 1(b). *Photomicrograph showing the phenomenon of agglutination at different time intervals i.e. 15, 30 and 45 min upon incubation of mouse spermatozoa with washed cells of S. marcescens (a,b,c), E. aerogenes (d,e,f), K. pneumoniae (g,h,i), E. coli (j,k,l) and S. aureus (m,n,o) (Original magnification X400)*

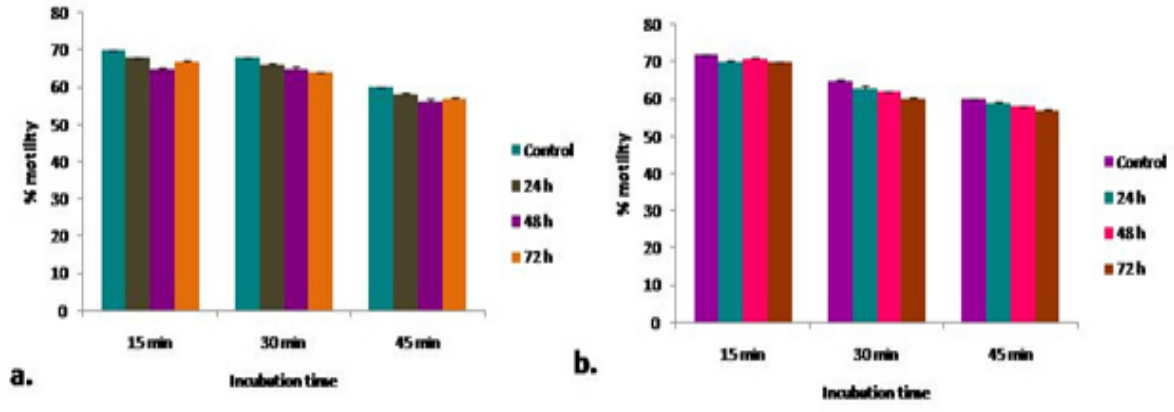


Fig 2. *Effect on sperm motility at different hours of incubation with 24/48/72h old culture of S. pyogenes (a), P. mirabilis (b) [Data represents mean ± SD]*

Viability

When the effect of these uropathogens on sperm viability was evaluated, the spermatozoa appeared as either pink stained or unstained (Figure 3). In comparison to control, upon incubation of mouse spermatozoa with the washed cells of sperm agglutinating (SA) microorganisms *viz. S. marcescens/E. aerogenes/K. pneumoniae/E. coli/S. aureus*, it was observed that all the microorganisms were capable of causing 100% sperm death (Figure 3). On the other hand, incubation of spermatozoa with washed cells of non sperm agglutinating (NSA) microorganisms *viz. S. pyogenes/P. mirabilis* did not show any reduction in sperm viability and were comparable to control spermatozoa (Figure 3).

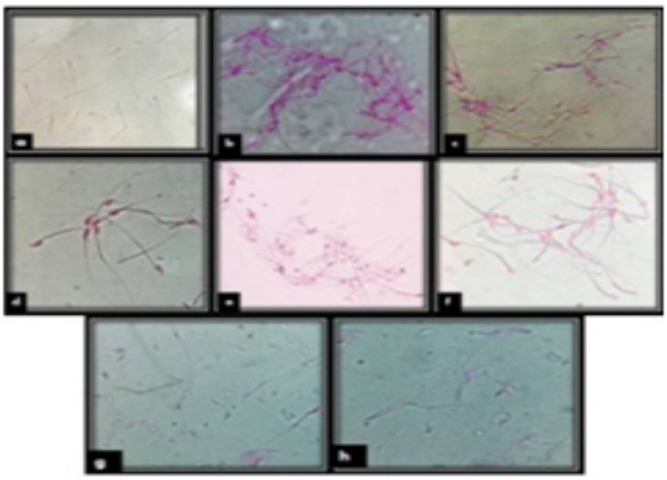


Fig 3. *Photomicrograph showing viability of mouse spermatozoa upon incubation with PBS (a), SA microorganisms viz. S. marcescens (b), E. aerogenes (c), K. pneumoniae (d), E. coli (e), S. aureus (f), NSA microorganisms viz. S. pyogenes (g), P. mirabilis (h). (Original magnification X400)*

Morphology

Scanning electron microscopy was carried out to examine the morphological alterations, if any, in mouse spermatozoa after incubation with these uropathogenic bacteria. The results revealed that in control sample where spermatozoa were incubated with PBS, normal mouse spermatozoa characterized by a falciform-shaped with apical hook could be observed. Further, the morphology of the neck and tail was also normal **(Figure 4a)**.

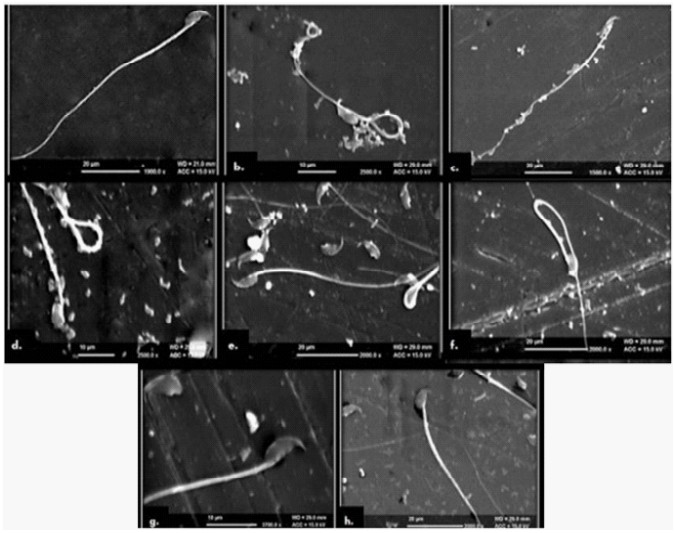


Fig 4. *Scanning electron micrographs showing mouse spermatozoa upon incubation with PBS (a), and washed cells of SA microorganisms viz. S. marcescens (b) E. aerogenes (c) K. pneumoniae (d), E. coli (e), S. aureus (f) and NSA microorganisms viz S. pyogenes (g), P. mirabilis (h).*

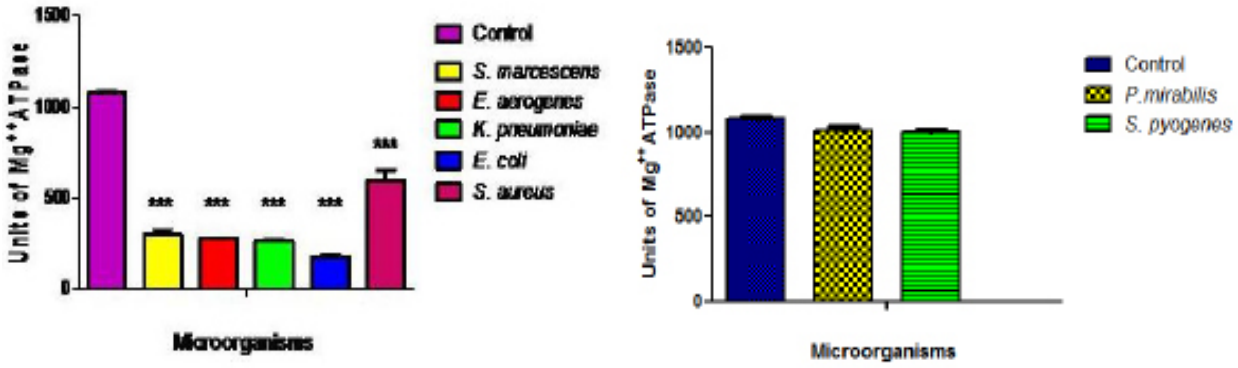
However, when the mouse spermatozoa were incubated with washed cells of sperm agglutinating (SA) microorganisms *viz*, *S. marcescens*, *E. aerogenes*, *K. pneumoniae*, *E. coli* and *S. aureus*, it was observed that all the sperm agglutinating microorganisms could adhere to spermatozoa and lead to considerable changes in the spermatozoa structure. The incubation with *S. marcescens* showed attachment of *S. marcescens* to the head, neck, midpiece and tail of spermatozoa and was also accompanied by curling of tail **(Figure 4b)**. *E. aerogenes* adhered throughout the length of spermatozoa from head to tail **(Figure 4c)**. Adherence of *K. pneumoniae* to sperm was also observed along with morphological alterations in terms of curling of spermatozoa tail **(Figure 4d)**. *E. coli* adhered to tail region of mouse spermatozoa and resulted in sperm decapitation **(Figure 4e)**. *S. aureus* adhered to mid region of spermatozoa and caused spermatozoa tail curling **(Figure 4f)**. Upon incubation with washed cells of NSA microorganisms (*S. pyogenes/P. mirabilis*), no adherence or morphological alterations could be observed in mouse spermatozoa and the morphology was comparable to control **(Figure 4g-4h)**.

Mg^{2+} Dependent ATPase Activity

When the effect of different uropathogens on Mg^{2+}dependent ATPase activity of mouse spermatozoa was checked, the results revealed that SA microorganisms could drastically inhibit Mg^{2+} dependent ATPase activity.

In case of incubation of sonicated spermatozoa with washed cells of SA microoganisms *viz. S. marcescens, E. aerogenes*, *K. pneumoniae*, *E. coli* and *S. aureus,* there was sharp reduction in ATPase units from 1082.24±5.4 (control) to 303.39±2.4, 278.64±2.49, 262.67±9.9, 176.04±1.4, 593.24±3.7 respectively, within 1h of incubation ($p<0.001$) **(Figure 5 a)**.

However, when the sonicated spermatozoa were incubated with washed cells of *S. pyogenes/ P. mirabilis*, no inhibition in Mg^{2+} dependent ATPase activity of mouse spermatozoa could be observed and the results were comparable to control. The Mg^{2+} ATPase units on incubation with washed cells were found to be 1009.18±6.2 (*S. pyogenes)* and 1013.97±19.7 (*P. mirabilis*) ($p>0.05$) (Figure 5 b).



a. b.

Fig 5. *Effect on Mg^{2+} dependent ATPase activity of mouse spermatozoa upon incubation with washed cells of SA microorganisms (a), washed cells of NSA microorganisms (b) [Data represents mean ± SD; $p<0.05$ (*), $p<0.01$(**), $p<0.001$(***)].*

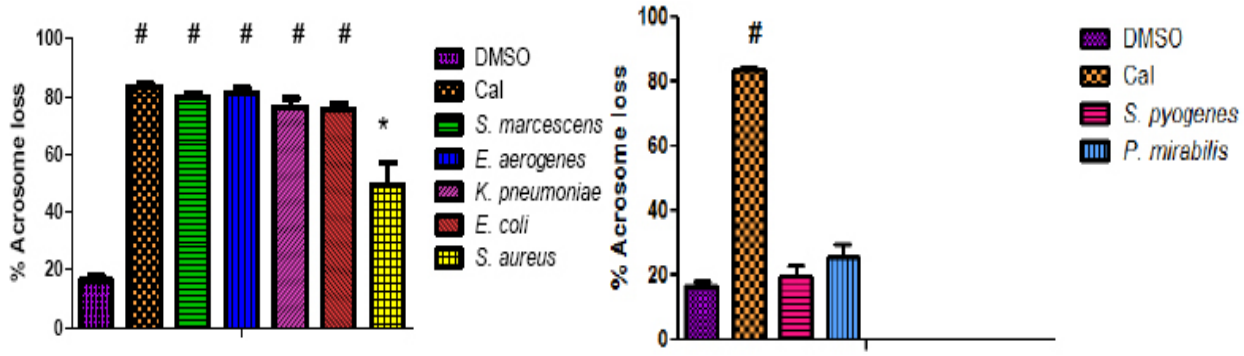
Acrosome Status

To check the effect of these microorganisms on acrosome status, mouse spermatozoa were incubated with washed cells of SA microorganisms or washed cells of NSA bacteria followed by PSA-FITC staining and observed through fluorescent microscopy.

The results showed that percentage of reacted spermatozoa in DMSO (negative control) was 16.33±1.52 whereas, percentage of reacted spermatozoa in case of sample treated with CaI (positive control) was 83.33±1.1 **(Figure 6a)**.

The percentage of acrosome reacted spermatozoa as compared to DMSO control was found to be significantly higher when spermatozoa were incubated with washed cells of SA microorganisms. In case of *S. marcescens*, 83.3±1.64% of spermatozoa was found to be reacted. Acrosome reaction to an extent of 76.3±1.5% and 81.3±1.5% was also apparent in spermatozoa treated with *K. pneumoniae* and *E. aerogenes*, respectively **(Figure 6a, 7)**. *E. coli* and *S. aureus* also showed 75.7±2.1%, 49.0±8.0% percent acrosome reactions respectively.

However, there were no significant differences in the acrosome reaction rate between spermatozoa incubated with DMSO or with NSA microorganisms. The percentage of spermatozoa that endured acrosomal reaction on incubation with washed cells of NSA bacteria was about 19.6 ± 3.2 (*S. pyogenes*) and 25.67±4.2 (*P. mirabilis*) **(Figure 6b, 7)**.



a. b.

Fig 6. *Percentage of acrosome reacted spermatozoa in case of incubation with washed cells of SA microorganisms a), washed cells of NSA microorganisms (b) as compared to DMSO and CaI [Data represents mean ± SD; p<0.05 (*), p<0.01(**), p<0.001(#)]*

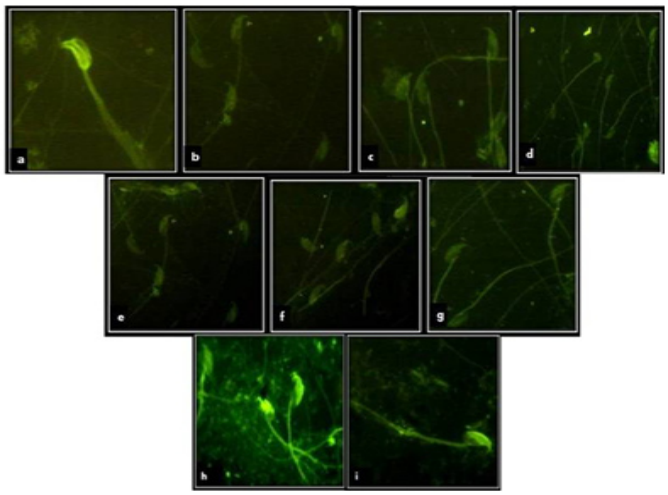


Fig 7. *Photomicrograph showing acrosomal status of spermatozoa. Acrosome intact spermatozoa upon incubation with DMSO (a), Acrosome reacted spermatozoa upon incubation with CaI (b), SA microorganisms viz. S. marcescens (a), E. aerogenes (b), K. pneumoniae (c), E. coli (d), S. aureus (e) and Acrosome intact spermatozoa upon incubation with NSA microorganisms viz. S. pyogenes (h), P. mirabilis (i).*

Discussion

Sexually transmitted infections have long been linked with infertility caused either by impairing the morphology/function of spermatozoa or by inflammatory changes in the genital tract. The impact of various sexually transmitted bacteria such as *Chlamydia trachomatis, Neisseria gonorrhoeae and Ureaplasma urealyticum* on seminal parameters has been well established. But, the role of other uropathogens *viz. E. coli, Enterobacter* spp, *Proteus* spp, *Streptococcus* spp, etc. on the same still remains speculative. Hence, the present study provides the retrospect on the effect of specific bacterial pathogens on the fertilization potential of mouse spermatozoa.

In this *in vitro* study, the various uropathogenic microorganisms were incubated with mouse spermatozoa in order to determine any negative impact on sperm parameters. When the effect of these uropathogens grown for 24, 48 and 72h was observed on sperm motility, the results showed that the five strains

i.e. *S. marcescens, E. aerogenes, K. pneumoniae, E. coli and S. aureus* could significantly reduce sperm motility *via* agglutination. However, *S. pyogenes* and *P. mirabilis* did not affect the sperm motility at all. Several investigators have also demonstrated that microorganisms exert negative effect on sperm motility and thus, compromised the fertility potential of males (Sanocka- Maciejewska *et al*. 2005), whilst, some claimed no consistent effect on the same (Berktas *et al.* 2008).

In order to detect if uropathogens only affect motility of spermatozoa or they could also alter membrane integrity leading to sperm death, the effect of uropathogens on sperm viability was studied by means of dye exclusion method using eosin. It was apparently clear from the results that incubation of spermatozoa with SA microorganisms led to sperm death indicating their devastating effect on sperm motility along with viability as well. However, non sperm impairing microorganisms *S. pyogenes* and *P. mirabilis* did not cause any sperm death as sperm parameters remain unaffected.

Further, Piasecka *et al*. (2014) reported that certain microorganisms deteriorate the sperm quality, not only by negatively affecting the sperm parameters *viz.* motility and viability, but also cause morphological alterations in sperms. The results of the SEM in the present study, illustrated the successful adherence of all SA microorganisms to spermatozoa with profound morphological changes in terms of sperm decapitation and curling of spermatozoa tail. From these results we could assume that the adherence of bacteria to the spermatozoa might be one of the reasons behind the decreased spermatozoa motility, that results in agglutination. Li *et al.* (2018) also revealed through electron microscopy that a strain of *S. aureus* attaches to spermatozoa that was accompanied by numerous structural deteriorative alterations.

It is a well known fact that spermatozoa use cation-dependent ATPases that are responsible for the breakdown of ATP to release energy for flagellar contractile processes to maintain sperm motility (Li *et al.* 2016). The inhibition of these ATPases by any means results in a significant decrease in sperm motility (Kocak-Toker *et al.* 2002). From results of the effect of these microorganisms on Mg^{2+} dependent ATPase activity, a menacing relationship between sperm agglutinating microorganisms and Mg^{++}dependent ATPase activity was seen, whereas, the same remained unaltered in case of non sperm impairing microorganisms. Thus, it could be assumed that decrease in cation-dependent ATPase activity induced by these microorganisms might have interfered with the biochemical mechanisms that may be responsible for maintaining sperm motility, thereby, resulting in diminished sperm motility. Peralta-Arias *et al.* (2015) also showed that inhibition of the dynein-ATPase activity, an intracellular motor for sperm motility, may have led to lack of sperm motility.

When the impact of these SA/NSA microorganisms on sperm acrosome reaction was studied, it was noted that the percentage of reacted acrosome in spermatozoa incubated with SA microorganisms was significantly higher than DMSO control and comparable to the premature acrosomal loss induced in CaI control. On the other hand, non sperm impairing microorganisms did not alter the percentage of spermatozoa with reacted acrosome and the results were comparable to DMSO control. These results indicated that the presence of SA microorganisms could negatively influence the membrane function of spermatozoa, thereby, resulting in premature acrosomal loss, which could be a consequence of either untimely sperm activation or shattering of sperm membranes that might interfere with further sperm function. In a similar study by Bar *et al.* 2008, it has been reported that *Campylobacter fetus* induces premature acrosomal loss in rat spermatozoa.

Thus, it could be concluded from the present study that the uropathogenic microorganisms present in male genitourinary tract/semen have perilous detrimental effect on sperm parameters thereby, might be playing an important role in causing male infertility.

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