Alterations in human sperm parameters as a corollary of incubation with various uropathogenic microorganisms.

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Abstract

Purpose: To analyze the impact of different uropathogenic microorganisms on various sperm parameters those are generally used to determine male fertilizing potential.

Method: The standard strains viz. Serratia marcescens, Klebsiella pneumoniae, Enterobacter aerogenes, Candida albicans, Proteus mirabilis, Streptococcus pyogenes, Klebsiella oxytoca were checked for their impact on sperm motility, viability, morphology, Mg++ ATPase activity and acrosome status of spermatozoa.

Results: S. marcescens, K. pneumoniae, E. aerogenes compromised sperm motility via agglutination and C. albicans impaired motility without agglutination. Further, these strains could also reduce viability resulting in sperm death. These strains induced morphological defects in spermatozoa as evident by electron microscopic studies. Incubation of spermatozoa with these uropathogenic strains led to reduction in Mg2+ dependent ATPase activity and also resulted in premature acrosomal loss. On the other hand, P. mirabilis, S. pyogenes, K. oxytoca did not alter any of these parameters.

Conclusion: It can be concluded from the present study that certain uropathogenic strains can induce detrimental effects on spermatozoa thereby reducing its fertilizing ability.

Keywords: Sperm motility, Viability, Morphology, Mg++ Atpase activity, Acrosome reaction, Uropathogens.

Introduction

Infections of the urogenital tract are believed to be of considerable significance in determining fertility outcome in males. Globally, they account for about 15% of these male infertility cases [1]. These infections lead to perturbation in male genital tract, thereby, damaging important biological functions of the organs of reproductive system. They can employ number of mechanisms either direct negative impact seminal fluid because of decline in the spermatozoa number, inhibition of motility, alterations in morphology and fertilizing capacity or indirect impact via impairment of spermatogenesis due to damage to testis, autoimmune conditions caused by inflammation, secretory dysfunction of accessory sex glands, leukocytospermia along with its impact on semen parameters, etc [2]. Amongst these, impairment of sperm parameters as corollary of semen infection plays a detrimental role. The source of microorganisms in semen might be sexually transmitted infections such as Neisseria gonorrhoeae and Chlamydia trachomatis or urinary tract infections such as Escherichia coli [3]. Amongst sexually transmitted microorganisms, U. urealyticum can deteriorate semen quality and fertilizing potential of spermatozoa by negatively altering the sperm motility, density and morphology [4]. Chlamydia infection is also linked with impaired sperm characteristics viz. motility, morphology and can also induce apoptosis of spermatozoa [5]. Amongst uropathogens, E. coli and S. aureus is also associated with severe negative effects on sperm parameters with respect to sperm motility, viability, acrosome reaction, morphology. In context to these reports, in the present study, an attempt was made to study the impact of other uropathogens on sperm parameters.

Materials and Methods

Microorganisms

The standard uropathogenic strains of viz. Serratia marcescens (MTCC 7641), Enterobacter aerogenes (MTCC 7324), Klebsiella pneumoniae (MTCC 4030), Candida albicans (MTCC 1637), Proteus mirabilis (MTCC 425), Streptococcus pyogenes (MTCC 1924), Enterococcus faecalis (MTCC 439) and Klebsiella oxytoca (MTCC 2275) used in the present study were procured from Microbial Type Culture Collection, Institute of Microbial Technology, Sector-39 Chandigarh, India. The bacterial strains were grown in Brain heart infusion (BHI) broth and C. albicans was cultivated in Sabouraud's dextrose broth (SDB). For long term storage, the strains were kept at -80°C in BHI/SDB broth containing 40% glycerol. For long term storage, the strains were kept at -80°C in BHI/SDB broth containing 40% glycerol.

Semen sample

Semen samples were collected by masturbation following sexual abstinence of 3-5 days at the clinical site (Department of Obstetrics and Gynecology, PGIMER, Chandigarh, India), from men undergoing semen analysis as a part of fertility assessment. All samples were retrieved into sterile plastic containers and allowed to liquefy for 30-45 min at 37°C. A
routine semen analysis was then performed according to World Health Organization (WHO) guidelines. Only those ejaculates that presented with normal semen parameters according to WHO criteria were used in the present study [6]. This study was approved by Institutional Ethics Committee (PUEIC), Panjab University, Chandigarh vide letter no IEC No130A-1 dated 29.10.2014.

Impact of uropathogenic strains on structural and functional parameters of spermatozoa in vitro motility

To assess the effect of standard strains on sperm motility, 100 μl each of the 24/48/72 h old culture and semen sample were mixed and incubated at 37°C. The uninfected semen suspension served as control. At different time intervals i.e. 30 min, 1, 2, 4 h, 10 μl of the mixture was placed on a clean glass slide, covered with a coverslip and observed under bright field microscope at a magnification of 400X (Olympus India Pvt. Ltd.). Further, to determine whether the changes in motility of spermatozoa was the property of washed cells (WC) or cell free supernatant (CFS), 100 μl of semen sample was incubated with either washed cells or CFS of all the cultures and observed for motility at different time intervals.

The results showed that 72 h old cultures have maximum inhibitory effect on sperm motility. Moreover, decline in sperm motility via agglutination was the property of washed cells, whereas decrease in sperm motility without agglutination found to be associated with CFS, hence for subsequent in vitro experiments; Washed cells of sperm agglutinating (SA) microorganisms, CFS of sperm immobilizing (SI) and both washed cells and cell free supernatant of non-sperm immobilizing-non sperm agglutinating microorganisms were used.

Viability

In order to assess the impact of these microorganisms on viability of human spermatozoa, dye exclusion assay using Eosin Y (0.5%), as indicator dye, was carried out. For this, equal volumes (100 μl) each of the CFS/WC of different microorganisms and semen sample were mixed and incubated at 37°C. After all the spermatozoa were immobilized, a wet preparation, consisting of by thoroughly mixed 10 μl each of reaction mixture and Eosin Y, was prepared and observed under bright field microscopy. As control, BHI broth was mixed and incubated with semen sample. The results were interpreted as pink stained (dead) and unstained (live) spermatozoa.

Morphology

The effect of different pathogens on morphology of spermatozoa was evaluated using scanning electron microscopy (SEM) using the protocol of Hafez and Kanagawa [7]. In brief, 200 μL of washed sperm suspension (containing 40 × 10⁶ spermatozoa/mL) and 200 μL of WC/CFS of different microorganisms were mixed and incubated for 1 h. This was followed by fixation using 4 ml of 2.5% phosphate buffered glutaraldehyde and incubation for 30 min. The samples were centrifuged and washed twice in PBS (50 mM, pH 7.2). Simultaneously, control was set which consisted of PBS as an alternative of WC/CFS. A drop of fixed and washed spermatozoa was placed on a silver painted adhesive tape mounted on brass stubs and air dried. 100Å gold coating was done on Jeol fine coat ion sputter (JFC-1100). The specimens were examined using Jeol scanning microscope (JSM-6100, Japan) at Sophisticated Analytical Instrumentation Facility (SAIF), Panjab University, Chandigarh.

Mg⁺⁺ dependent ATPase activity

To explicate if decline in motility of spermatozoa is a consequence of decrease in ATPase activity of spermatozoa, the effect of SA/SI/NSA-NSI microorganisms on activity of Mg⁺⁺ dependent ATPases activity was checked. The washed human spermatozoa (1 × 10⁸/ml) were sonicated in Tris-HCl (0.2 M, pH 7.6) and incubated with 200 μl Tris- Cl buffer (0.2 M, pH 7.6), 200 μl of MgCl₂ (5 mM), 200 μl of ATP (6 mg/ml) and 200 μl of WC/CFS of different microorganisms. As control, mixture containing PBS in place of cell free supernatant was used. These reaction mixtures were incubated at 37°C for 1 h. This was followed by addition of 1 mL of cold 10% Trichloroacetic acid (TCA) to stop the reaction and incubation at 4°C, overnight for protein precipitation. Inorganic phosphorus (Pi) released was evaluated in accordance to the procedure described by Boyce et al. [8]. One unit of ATPase was expressed as μ moles of the Pi released after 1 h of incubation.

Acrosome reaction

The impact of SA/SI/NSA-NSI microorganisms on sperm acrosome status, an indicator of functional status of spermatozoa, was also assessed. For this, the washed sperm pellet was capacitated by suspending in Ham’s F-10 (containing HEPES and 1% human serum albumin) and incubating for 3 h at 37°C. After incubation, spermatozoa were collected by centrifugation (500 g for 10 min) and motility was checked. To calculate the number of spermatozoa undergoing acrosome loss, aliquots of only highly motile spermatozoa were incubated with 0.1% of DMSO (negative control)/10 μM Calcium ionophore A23187 (positive control)/WC/CFS and incubated for 1 h at 37°C. This was followed by staining of spermatozoa with PSA-FITC, for which, spermatozoa in above mentioned aliquots were washed twice with 10 ml of 0.9% sodium chloride and smeared on clean glass slide. The smear was fixed in 95% ethanol for 30 min, air dried and washed with distilled water. It was stained with 25 mg/ml PSA-FITC in PBS (pH 7.4) for 4 h at 4°C. The slides were rinsed in water to remove excess PSA-FITC. The slides were air dried and were covered by 30 μL of anti-fading medium (25 mg/ml 1:4-diazabicyclo[2, 2, 2]octane) to prevent photo-bleaching and at least 200 spermatozoa were counted using a fluorescence microscope (Nikon, Tokyo, Japan). The results were inferred as

- Acrosome intact (AI): Bright uniform fluorescence on more than half the head of a spermatozoon
- Acrosome reacted (AR): No fluorescence or fluorescent band limited to the equatorial segment of head of spermatozoon.

Results

Motility

To assess the effect of uropathogens on sperm motility, 24, 48 and 72 h old cultures were incubated with spermatozoa and the motility was observed at different time intervals viz. 30 min, 1, 2 and 4 h. The results showed that S. marcescens, E. aerogenes and...
K. pneumoniae were found to exert a significant depressor effect on sperm motility via agglutination while significant decrease in sperm motility that followed incubation of spermatozoa with C. albicans was without agglutination (Figures 1 and 2). On the other hand, incubation of spermatozoa with 24/48/72 h old cultures of P. mirabilis/S. pyogenes/E. faecalis/K. oxytoca did not result in any negative influence on sperm motility at any of the specified time intervals (Figure 3). Further, maximum sperm impairing activity was exhibited by 72 h old cultures; hence, for further experiments 72 h old cultures were used. Moreover, the results indicated that only WC could agglutinate spermatozoa, while CFS failed to do so whereas sperm immobilization without agglutination was visualized only with CFS.

**Viability**

When the effect of these uropathogens on sperm viability was evaluated using eosin as the indicator dye, the results revealed that in comparison to control, SA/SI microorganisms were capable of inducing 100% sperm death. On the other hand, death was not evident in case of incubation of spermatozoa with NSA-NSI microorganisms (Figures 4-7).

**Morphology**

Scanning electron microscopy was carried out to examine the morphological alterations, if any, in spermatozoa after incubation with different microorganisms. The results showed that in control sample normal human spermatozoa characterized by regular oval shaped head could be observed. Further, the morphology of the neck and tail was also normal (Figure 8). However, incubation of spermatozoa with S. marcescens showed attachment of S. marcescens to the tail of spermatozoa and was also accompanied by morphological alterations in head. E. aerogenes adhered to sperm head and an
interesting morphological alteration i.e. the curling of tail in spermatozoa could be observed. Adherence of *K. pneumoniae* to sperm head was also observed along with morphological alterations in the head (Figure 9). *C. albicans* also resulted in morphological alterations in neck and mid piece as indicated by loosening and disruption of sperm membrane (Figure 10). Along with, SEM analysis carried out with WC of *C. albicans* did not result in any adherence. Upon incubation with washed cells/cell free supernatant of NSA-NSI microorganisms (*P. mirabilis*, *S. pyogenes*, *E. faecalis*/*K. oxytoca*), no adherence or morphological alterations could be observed in spermatozoa and the sperm morphology was comparable to control (Figure 11).

**Mg**\(^{++}\) dependent ATPase activity

When the effect of different uropathogens on Mg\(^{++}\)-dependent ATPase activity of human spermatozoa was checked, the results revealed there was significant reduction in ATPase units from 1142.91 ± 8.1 (control) to 390.81 ± 7.01, 269.06 ± 7.69, 390.40 ± 9.9 and 403.19 ± 11.31. *S. marcescens*, *E. aerogenes* and *K. pneumoniae* and *C. albicans*, respectively, within 1 h of incubation (p<0.001). In case of incubation of sonicated spermatozoa with washed cells/supernatant of *P. mirabilis*, *S. pyogenes*, *E. faecalis*/*K. oxytoca*, no inhibition in Mg\(^{++}\) dependent ATPase of human spermatozoa could be observed and the results were comparable to control. The Mg\(^{++}\) ATPase
units on incubation with washed cells and cell free supernatant were found to be 1126.026 ± 8.17 and 1113.37 7.97 (P. mirabilis), 1128.942 ± 6.14 and 1132.13 ± 10.1 (S. pyogenes), 1134.132 ± 9.4 and 1120.16 ± 8.2 (E. faecalis), 1115.768 ± 8.49 and 1122.15 ± 13.1 (K. oxytoca), respectively (p>0.05).

Acrosome status

In an attempt to study the effect of SA/SI/NSA-NSI microorganisms on acrosome status, human spermatozoa were incubated with various microorganisms followed by PSA-FITC staining and observation through fluorescent microscopy. The results showed that percentage of reacted spermatozoa in DMSO (negative control) was 12.66 ± 1.52 whereas percentage of reacted spermatozoa in case of sample treated with Cal (positive control) was 90.66 1.6 (Figure 12). The percentage of acrosome reacted spermatozoa as compared to DMSO control was observed to be significantly higher when spermatozoa were incubated with washed cells of SA/SI microorganisms. In case of S. marcescens, 83.0% ± 2.1% of spermatozoa were found to be reacted. Acrosome reaction to an extent of 81.6% ± 5.1% and 87.5% ± 1.7% was also apparent in spermatozoa treated with K. pneumoniae and E. aerogenes, respectively (Figure 13). A statistically significant increase in percentage of acrosome reacted spermatozoa was also observed in case of C. albicans wherein 68.1% ± 2.8% of spermatozoa were found to be reacted (Figure 14). However, there were no significant differences in the acrosome reaction rate between spermatozoa incubated with DMSO or with NSA-NSI microorganisms. The percentage of spermatozoa that underwent acrosomal exocytosis on incubation with washed cells of bacteria was about 15.83 ± 1.7 (P. mirabilis), 16.1 ± 2.0 (S. pyogenes), 16.3 ± 2.51 (E. faecalis), 14.5 ± 1.32 (K. oxytoca) and with cell free supernatant was found to be 16.6 ± 1.51 (P. mirabilis), 16.5 ± 1.0 (S. pyogenes), 14.3 ± 1.52 (E. faecalis), 15.1 ± 0.76 (K. oxytoca) (Figure 15).

Discussion

Mammalian spermatozoon is a very specialized cell that does a number of biological activities such as movement, recognition of zona pellucida, acrosome reaction to eventually attain its final aim of fertilizing the oocyte [9]. Any agent that can elicit detrimental effect on these features of sperm

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of different microorganisms E. faecalis bacterial growth i.e. 72 h old culture showed maximum activity. These microorganisms also showed positive correlation with immobilization without agglutination. The inhibitory effect of sperm motility

sperm agglutination was the property of washed cells, whereas, immobilization was associated with cell free supernatant. Since bioactive component in SA strains was washed cells and in case of SI strains was cell free supernatant, hence, these components were used for further in vitro experiments.

Another important parameter which is used as an indicator of cellular membrane integrity of spermatozoa is viability [17]. As per WHO guidelines, if the percentage of non-motile spermatozoa is greater than 40, it is clinically imperative to confirm the ratio of viable spermatozoa [6]. Therefore, next experiment was undertaken to study if sperm impairing microorganisms could induce sperm death as well. On the basis of results, it was apparent that incubation of spermatozoa with SA and SI strains led to sperm death, however, NSA-NSI strains failed to do so.

Further an attempt was made to check any morphological defect in spermatozoa as a consequence of their incubation with bacteria. The micrographs generated through scanning electron microscopy revealed adherence of S. marcescens, E. aerogenes and K. pneumoniae with spermatozoa, thereby, leading to prominent morphological defects. C. albicans was also capable of inducing profound morphological alterations in the neck region and also led to disruption of plasma membrane in structure of spermatozoa but without adhering to the spermatozoa. However, NSA-NSI strains did not elicit any detrimental effect on sperm parameters. The results further substantiated the fact that immobilization via agglutination by SA microorganisms was due to adherence of cells to spermatozoa. Similar to the findings obtained in present study, earlier Diemer et al. [18] have also reported that immobilization of spermatozoa by E. coli is attributed to tight adhesions which is followed by damage to the structure of spermatozoa as viewed by SEM analysis. Also, the morphological alterations in spermatozoa in case of C. albicans have also been demonstrated through electron microscopy by Tian et al. [14].

It is well known that after entering the female reproductive tract, male gamete senses the environment and adapts its motility, which is controlled partially by ATP. It possesses several ATPases, each of which is dependent on the different cations such as Na\(^{+}\), K\(^{+}\), Mg\(^{++}\), that account for the breakdown of ATP to release energy for flagellar contractile processes [19]. The inhibition of these ATPases by any means results in a significant decrease in sperm motility. To address the plausibility of involvement of cation dependent ATPases in microorganism induced sperm impairment, the effect on Mg\(^{++}\) dependent ATPase was studied. From the results, a negative correlation between the SA and SI microorganisms and Mg\(^{++}\) dependent ATPase activity was derived, whereas, the same remain unaltered in case of NSA-NSI strains. Thus, it could be anticipated that microbe induced decrease in cation dependent ATPase activity might have interfered with the biochemical mechanisms that are responsible for maintaining sperm motility, hence, resulting in diminished sperm motility.

Besides these structural parameters, an important functional parameter in terms of acrosome reaction was determined. It was noted that the percentage of intact acrosome in spermatozoa incubated with S. marcescens, E. aerogenes, K. pneumoniae and C. albicans was notably lesser as compared to control samples.
But, the NSA-NSI microorganisms did not alter the percentage of spermatozoa with intact acrosome. This indicated that presence of such microorganisms could negatively influence the membrane function of spermatozoa resulting in premature acrosomal loss, thereby, leading to diminished sperm parameters. The negative influence of bacteria on sperm acrosome status has also been demonstrated by el Mulla et al. [20].

**Conclusion**

The conclusionary remarks of the study are that certain uropathogens viz. *S. marcescens*, *E. aerogenes*, *K. pneumoniae* and *C. albicans* either directly or by releasing soluble factors could negatively influence structural and functional parameters of spermatozoa in vitro, thereby, reducing the fertilizing sperm potential.

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**References**


