Efficacy of two sperm preparation techniques in reducing non-specific bacterial species from human semen

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ABSTRACT

CONTEXT: Artificial reproductive techniques using seminal preparations with bacteria may cause pelvic inflammatory disease and its sequalae. **AIMS:** To assess efficacy of two sperm preparation techniques to clear bacteria and the effect of bacteriospermia on sperm recovery rates. **SETTINGS AND DESIGN:** A descriptive cross-sectional study was carried out among males of subfertile couples. **SUBJECTS AND METHODS:** Semen samples were randomly allocated into swim-up method (group S, $n = 68$) and density gradient method (group D, $n = 50$) for sperm preparation. Seminal fluid analysis and bacterial cultures were performed in each sample before and after sperm preparation. **STATISTICAL ANALYSIS:** McNemar's chi-squared test and independent samples *t*-test in SPSS version 16.0 were used. **RESULTS:** Organisms were found in 86 (72.88%) out of 118 samples, before sperm preparation; *Streptococcus* species ($n = 40$, 46.51% of which 14 were Group D *Streptococcus* species), Coagulase negative *Staphylococcus* species ($n = 17$, 19.76%), *Staphylococcus aureus* ($n = 13$, 15.11%), *Coliform* species ($n = 11$, 12.79% of which 09 were *Escherichia coli*) and *Corynebacterium* species ($n = 5$, 5.81%). There was a statistically significant reduction of culture positive samples in raw vs. processed samples; in group S, 49 (72.05%) vs. 16 (23.52%) and in group D, 37 (74%) vs. 18 (36%). In group S and D, mean (SD) recovery rates of culture positive vs. culture negative samples were 39.44% (SD-14.02) vs. 44.22% (SD-22.38), $P = 0.39$ and 52.50% (SD-37.16) vs. 49.58% (SD-40.32), $P = 0.82$ respectively. **CONCLUSIONS:** Both sperm preparation methods significantly reduced bacteria in semen, but total clearance was not achieved. Sperm recovery rate was not affected by bacteriospermia.

KEY WORDS: Bacteriospermia, density gradient, sperm recovery rate, swim-up

INTRODUCTION

Bacteriospermia is the presence of bacteria in seminal fluid. Detection of bacteria in semen does not necessarily signify infection as bacteriospermia may represent contamination during sample collection, bacterial colonization of the distal segment of the urethra or infection.[1-3] Testes, epididymis, vas deferens, ejaculatory ducts, and the proximal portion of the urethra are usually devoid of bacteria in a normal male. Fluid from the vas deferens of men undergone vasectomy has uniformly yielded negative culture results.[4] Semen that passes through the genital tract is routinely contaminated by gram-positive bacteria, usually *Staphylococcus* species, *Streptococcus* species and *Corynebacterium* species.[5] A significant growth of bacteria in culture; $>10^3$ colony forming units/ml (CFU/ml), detection of *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Ureaplasma urealyticam* and/or significant leukocytospermia ($>10^6$ peroxidase-positive leukocytes/ml) may indicate an infection.[5,6] Prevalence of bacteriospermia and types of organisms found in seminal fluid vary depending on the populations studied and methods used for the detection of bacteria. When polymerase chain reaction (PCR)-based bacterial detection methods were used, the prevalence of bacteriospermia was significantly higher[7,8] compared to other methods used in different studies.[9,10] A recent study using molecular biological

techniques showed that >10^4 CFU of bacteria/ml in 66% of asymptomatic subfertile men. But when using routine culture methods, significant growth was found only in 27% in the same population.[11] *Chlamydia trachomatis, Mycoplasma hominis, Mycoplasma genitalium, Ureaplasma urealyticum, Ureaplasma parvum,* and *Gardnerella vaginalis* are frequently found organisms in semen when specialized culture media and molecular biological techniques were used.[12,13]

According to Diemer *et al*., bacteriospermia may affect fertility and account for 15% of cases of male subfertility.[14] Infectious processes can impair fertility by different mechanisms, including male accessory sex gland dysfunction, triggering of anti-sperm antibody production, deterioration of spermatogenesis, impairment of sperm function, obstruction of the excurrent ductal system, phagocytosis, and cytokine-mediated destruction by leukocytes.[5,15,16]

Intrauterine insemination (IUI) is an artificial reproductive technique, which is widely used to treat factor subfertility. IUI with contaminated semen may cause pelvic inflammatory disease and its sequalae in women. IUI bypasses the cervical mucus and thus may be expected to have a higher incidence of infections. Stone *et al*., found that positive results from peritoneal cultures in five of nine women after IUI with washed sperm. But none of these women demonstrated clinical infection.[17] The incidence of clinical infection after IUI is low. The incidence of infection after IUI with no antibiotic cover and without any antibiotics added to the semen processing medium, varied 1.83 to 2.1 per 1000 patients.[18] Therefore, effective semen processing procedures should be employed to remove bacteria from semen.[19] One method of clearing the bacteria from semen is addition of antibiotics to the sperm processing media.[20] Penicillin and streptomycin are the widely used antibiotics. But some manufacturers do not provide them in a ready-to-use form. Also, there is no consensus on beneficial effects over harm to the sperm from the use of antibiotics. Use of sterile techniques in sperm processing would help to minimize or eliminate bacteria from the post-wash sperm samples.

Swim up and density gradient sperm preparation techniques vary greatly in terms of recovery rates, motility, morphology, and degree of DNA damage.[21,22] These parameters influence the fertilization rates following IUI. The ability of sperm preparation techniques to clear bacterial species from the seminal fluid and effect of bacteriospermia on recovery rates of sperms are important aspects of sperm preparation. The objectives of this study were to assess the efficacy of swim up and density gradient techniques in clearing non-specific bacteria from seminal plasma and the effect of bacteriospermia on recovery rates of sperms in males of subfertile couples.

SUBJECTS AND METHODS

A descriptive cross-sectional study was carried out from June 2012 to January 2013. Ethical clearance was obtained from the ethics review committee of the institute. All consenting males of subfertile couples were included in the study after considering the exclusion criteria [Figure 1]. Semen samples were collected into sterile wide-mouthed polystyrene containers, after two to seven days of sexual abstinence. Males were advised to pass urine half an hour prior to the collection of the sample, to wash their hands and penis thoroughly using soap, rinse away soap and dry with clean disposable towels and not to use any lubricant or saliva at the time of sample collection by masturbation. They were explained the precautions to avoid contamination and spillage and advised to hand over the sample to the laboratory immediately after collection. Samples were randomly allocated into two groups by means of simple random sampling. In the first group (Group S) sperm preparation was done with swim-up method and in the second group (Group D) sperm preparation was done with density gradient method. Assessment of volume, sperm count, and motility was performed in each sample before and after the sperm preparation, according to the WHO guidelines.[23] Aliquots (0.5 ml each) from the initial raw semen sample and processed sample were set aside for bacterial culture. Samples were sent to the microbiology laboratory immediately after sperm processing, for the microbiological culture.

Sperm preparation by swim-up and density gradients methods were done according to the procedures given in WHO guidelines, without any antibiotics added to the sperm preparation medium. One millilitre of the initial semen sample was used to process the sperms. Culture of seminal fluid samples was performed, within 2 hours of collection. The samples were inoculated in Blood Agar, Chocolate Agar and McConkey Agar, using a calibrated loop. The inoculated samples were incubated overnight at 37°C in normal air with 5% CO_2 for 24 hours. Samples which showed more than one type of bacterial species in the culture media were excluded from the study. All the procedures were carried out in strict aseptic conditions.

- Men having had antibiotic treatment within the past three months
- Samples with sperm count <10×10^6/ml and/ or volume <1 ml
- Samples which showed more than one type of bacterial species in the culture media

Figure 1: Exclusion criteria used in recruitment

Data analysis was done with the use of SPSS version 16.0 software. Comparison of bacterial growth between the initial samples and the processed samples was done using the McNemar's chi-squared test. Sperm recovery rates between the sperm preparation techniques were compared using independent samples *t*-test.

RESULTS

The mean age of the study population was 34.2 years (Range 23-41 years). Out of 134 semen samples cultured, 102 (70.83%) showed presence of bacterial species in semen samples. Mixed growth of bacteria was observed in 16 samples, which were excluded from the study to eliminate the errors due to contamination. Semen samples with growth of a single organism (n = 86, 72.88%) and semen samples without any growth in culture media (n = 32, 27.11%) were included in the study, with the total sample size of 118. Organisms found were *Streptococcus* species (n = 40, 46.51% of which 14 were Group D *Streptococcus* species), Coagulase negative *Staphylococcus* species (n = 17, 19.76%), *Staphylococcus aureus* (n = 13, 15.11%), *Coliform* species (n = 11, 12.79% of which 09 were *Escherichia coli*) and *Corynebacterium* species (n = 5, 5.81%).

In group S, a positive bacterial growth was seen in 49 (72.05%) before the sperm preparation and only 16 (23.52%) after sperm preparation ($P < 0.001$). The specific organisms involved before and after sperm preparation were *Streptococcus* species excluding group D Streptococci 17 (25%) vs. 09 (13.23%): $P = 0.005$, Group D *Streptococcus* species in 05 (7.35%) vs. 01 (1.47%): $P = 0.046$, Coagulase negative *Staphylococcus* species in 10 (14.70%) vs. 02 (2.94%): $P = 0.005$, *Staphylococcus aureus* in 07 (10.29%) vs. 00 (0.00%): $P = 0.008$, *Coliform* species excluding *Escherichia coli* in 01 (1.47%) vs. 01 (1.47%), *Escherichia coli* in 06 (8.82%) vs. 03 (4.41%): $P = 0.083$, and *Corynebacterium* species in 03 (4.41%) vs. 00 (0.00%): $P = 0.083$. McNemar's chi-squared test was used for the comparison [Table 1].

In group D, 37 samples (74%) showed a positive bacterial culture before the sperm preparation and only 18 samples (36%) were found to have a bacterial growth in culture media after sperm preparation ($P < 0.001$). The specific bacteria found before and after preparation of semen samples were *Streptococcus* species excluding group D Streptococci (9, 18%) vs. 05 (10%): $P = 0.046$, Group D *Streptococcus* species in 09 (18%) vs. 06 (12%): $P = 0.083$, Coagulase negative *Staphylococcus* species in 07 (14%) vs. 03 (06%): $P = 0.046$, *Staphylococcus aureus* in 06 (12%) vs. 02 (4%): $P = 0.046$, *Coliform* species excluding *Escherichia coli* in 01 (2%) vs. 00 (00%): $P = 0.317$, *Escherichia coli* in 03 (6%) vs. 01 (2%): $P = 0.157$ and *Corynebacterium* species in 02 (4%) vs. 01 (2%): $P = 0.317$. McNemar's chi-squared test was used for the comparison [Table 1].

Recovery rates of sperms in culture positive vs. culture negative samples were 39.44% (SD-14.02) vs. 44.22% (SD-22.38) in group S, P = 0.39 and 52.5% (SD-37.16) vs. 49.58% (SD-40.32), P = 0.82 in group D respectively. There was no significant deference between culture positive and culture negative raw samples with regard to volume, concentration, and percentage of progressive motile sperms in either group. Independent samples *t*-test was used for the comparison [Table 2]. Calculation of the recovery rate was as follows:

$$\text{Total motile sperm recovery rate}(\%) = \frac{(\text{Volume} \times \text{Sperm concentration} \times \text{Sperm motility}) \text{ in the processed sample}}{(\text{Volume} \times \text{Sperm concentration} \times \text{Sperm motility}) \text{ in the raw sample}} \times 100$$

DISCUSSION

Most frequently found bacterial species in our study were *Streptococcus* species, Group D Streptococci, Coagulase negative *Staphylococcus* species, *Staphylococcus aureus*, *Escherichia coli* and *Corynebacterium* species. But when PCR based methods were used, gram positive anaerobes (*Peptoniphilis, Anaerococcus, Finegoldia, Peptostreptococcus* species) were isolated as the most prevalent bacterial species in seminal fluid.[7-9] Therefore, type of organisms isolated in seminal fluid varies according to the method used for detection of bacteria. The prevalence of bacteriospermia among subfertile males has shown to be 25-100%.[10,24-26] It is mainly determined by the population studied and method used to identify the bacterial organisms. Organisms identified in our study are known to reduce the quality of seminal fluid.[27-30] But in our study, there was no significant difference between culture positive and culture negative samples in respect to the volume, percentage progressive motility, sperm concentration, and recovery rates. Effect of bacteriospermia on clinical pregnancy rates needs further evaluation.

Sperm preparation techniques, allowing higher sperm recovery and motility rates, have become very useful in the treatment of male infertility.[31] The recovery rates of density gradient method are higher compared with the swim-up method, which makes the density gradient method the preferred sperm preparation method regardless of the initial fresh sample concentration.[32] Density gradient centrifugation is known to clear bacteria from the seminal plasma.[19] Swim-up method is found to be more efficient in clearing bacteria from the seminal fluid compared to treatment with antibiotics of the male partner.[33] In our study, both swim-up and density

Table 1: Reduction of number of culture positive samples by sperm preparation using swim-up (Group S) and density gradient (Group D) methods. (*n*=118)

Organisms isolated	Group S (*n*=68)			Group D (*n*=50)		
	Raw samples (%)	Processed samples (%)	*P* value	Raw samples (%)	Processed samples (%)	*P* value
Overall species	49 (72.05)	16 (23.52)	<0.001**	37 (74)	18 (36)	<0.001**
<10^3 CFU	19	07		13	07	
10^3-10^5 CFU	26	08		20	09	
>10^5 CFU	04	01		04	02	
Streptococcus species excluding group D Streptococci	17 (25)	09 (13.23)	0.005**	09 (18)	05 (10)	0.046**
<10^3 CFU	06	02		03	02	
10^3-10^5 CFU	11	07		06	03	
>10^5 CFU	00	00		00	00	
Group D *Streptococcus* species	05 (7.35)	01 (1.47)	0.046*	09 (18)	06 (12)	0.083
<10^3 CFU	01	00		05	03	
10^3-10^5 CFU	03	01		01	01	
>10^5 CFU	01	00		03	02	
Coagulase negative *Staphylococcus* species	10 (14.70)	02 (2.94)	0.005**	07 (14)	03 (6)	0.046*
<10^3 CFU	07	00		02	00	
10^3-10^5 CFU	02	02		05	03	
>10^5 CFU	01	00		00	00	
Staphylococcus aureus	07 (10.29)	00 (0.00)	0.008**	06 (12)	02 (4)	0.046*
<10^3 CFU	03	00		01	01	
10^3-10^5 CFU	04	00		04	01	
>10^5 CFU	00	00		01	00	
Coliform species exluding *Escherichia coli*	01 (1.47)	01 (1.47)		01 (2)	00 (0)	0.317
<10^3 CFU	00	01		00	00	
10^3-10^5 CFU	01	00		01	00	
>10^5 CFU	00	00		00	00	
Escherichia coli	06 (8.82)	03 (4.41)	0.083	03 (6)	01 (2)	0.157
<10^3 CFU	01	02		00	00	
10^3-10^5 CFU	03	00		03	01	
>10^5 CFU	02	01		00	00	
Corynebacterium species	03 (4.41)	00 (0.00)	0.083	02 (4)	01 (2)	0.317
<10^3 CFU	01	00		02	01	
10^3-10^5 CFU	02	00		00	00	
>10^5 CFU	00	00		00	00	

***P* value less than 0.01, **P* value less than 0.05, CFU=Colony forming units, Culture results of the raw samples were considered in the comparison

gradient methods were found to be effective in clearance of non-specific bacterial species in seminal plasma. Total clearance of bacteria was not achieved in either method. In samples with *Streptococcus* species, excluding group D Streptococci, there was a significant reduction of bacteria by either method but complete clearance was not achieved. In samples with Group D *Streptococcus* species there was a significant reduction of bacteria with the swim-up method compared to the density gradient method. In samples with coagulase negative *Staphylococcus* species there was a significant reduction of bacteria by both methods, but complete clearance was not achieved. In samples with *Staphylococcus aureus*, density gradient method was capable of significantly reducing the number of post preparation samples, while swim-up method cleared the bacterium from all the samples. Even though both methods significantly cleared the non-specific bacterial species, the presence of bacteria in some post preparation samples may carry a risk of pelvic infection following intra uterine insemination (IUI). The incidence of pelvic infection following IUI with processed sperms is low. However, if pelvic inflammatory disease (PID) develops in these women that may further compromise fecundity.[34] *In vitro* fertilization (IVF) is also affected by presence of bacteria in seminal fluid. Contamination with seminal microorganisms may lead to oocyte degeneration, suboptimal fertilization rates and impaired embryonic development following IVF.[33,35]

Other than using sterile techniques, sperm preparation in antibiotics added media may further improve the ability to clear bacteria from seminal fluid. Enrichment

Table 2: Comparison of seminal fluid parameters and recovery rates between culture* positive and negative samples. (*n*=118)

Parameter	Group S (*n*=68)			Group D (*n*=50)		
	Culture positive samples (*n*=49) mean (SD)	Culture negative samples (*n*=19) mean (SD)	*P* value	Culture positive samples (*n*=37) mean (SD)	Culture negative samples (*n*=13) mean (SD)	*P* value
Volume (ml) of						
Raw samples	1.69 (0.54)	1.64 (0.52)	0.73	2.01 (1.05)	1.89 (0.96)	0.74
Processed samples	0.49 (.04)	0.5 (0.00)	0.55	0.50 (0.00)	0.50 (0.00)	0.53
Percentage progressive motile sperms (%) of,						
Raw samples	61.32 (14.62)	60.52 (13.82)	0.83	46.05 (20.20)	49.69 (19.87)	0.52
Processed samples	101.22 (8.57)	101.05 (4.58)	0.91	92.81 (17.00)	93.92 (21.91)	0.33
Sperm concentration (Millions/ml) of,						
Raw samples	59.40 (27.03)	54.78 (25.53)	0.52	31.35 (25.61)	26.55 (28.41)	0.51
Processed samples	44.83 (25.84)	41.36 (27.75)	0.63	27.87 (39.09)	16.82 (17.32)	0.84
Total motile sperm recovery rate (%)	39.52 (14.31)	44.32 (22.13)	0.39	52.50 (37.16)	49.58 (40.32)	0.82

*Culture results of the raw samples were considered in the comparison

of the sperm preparation media with Penicillin and Streptomycin is proven to be effective in reducing the bacterial organisms.[36] But it is well known that antibiotics are biologically active substances, which may probably affect the cell function. Antibiotics are added to the embryo culture media in IVF, to avoid contamination from micro-organisms. But evidence suggests that the absence of antibiotics in culture media is associated with an increase in embryo cell division. Indeed, the elimination of penicillin and streptomycin from the media resulted in an improved cleavage rate.[37] Faster cleaving embryos have been clearly demonstrated to be more capable of implantation in animal species.[38,39] A strong positive correlation was found between cleavage delay and chromosomal abnormalities.[40] The effect of antibiotics added media on clinical pregnancy rates is not clear with the available evidence. There is no universal agreement of adding antibiotics to the sperm preparation and/or embryo culture media in artificial reproductive techniques. Therefore, most centres in our set up, including the centre where the study was carried out, did not use antibiotic added media for sperm preparation. Therefore, more efficient methods should be implemented to improve the sperm preparation techniques to clear bacteria from the seminal plasma as the swim-up and density gradient techniques alone were inadequate to achieve complete clearance of the non-specific bacterial species from the seminal fluid.

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