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Original Research

Elimination of bacteria from semen using a combination of density gradient centrifugation and swim-up

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Abstract

Background: To evaluate the efficiency of bacterial clearance of different sperm preparation procedure. **Methods**: A total of 46 semen samples were collected in this study, each semen sample was divided into four equal parts (0.5 mL each). The first part was prepared by density-gradient centrifugation (D-group), the second part was prepared by swim-up (S-group), the third part was treated with density-gradient centrifugation combined with swim-up (D+S-group), and the fourth group was the original raw semen group (O-group) regarded as the control. After each semen preparation procedure, the O-group and suspensions were inoculated and incubated for bacterial identification and colony counting. **Results**: Initially only 8.7% (4/46) of samples were bacteria free. After processing, the bacterial clearance rates were 23.8% for D-group, 57.4% for S-group and 97.8% for D+S-group ($p < 0.001$). Multiple bacterial strains were observed in 37 samples with 117 different bacterial strains in all identified in the original semen. 44.4% of the staphylococci and 89.7% of streptococci were not eliminated in D-group. In S-group, the corresponding rates were 24.4% and 35.9%. In D+S-group there were no remaining strains of staphylococci and only 2.6% of streptococci remained. After the combined procedure, the number of bacterial colonies fell dramatically after processing. **Conclusion**: The combined D+S protocol appears to be substantially more efficient than either method alone in eliminating bacteria from semen samples.

Keywords: Semen; Bacteria; Density-gradient centrifugation; Swim-up

1. Introduction

Contamination of embryo culture medium can lead to arrest of embryo development and implantation failure. This is a rare condition, with the incidence to be 0.35%–0.69% [1–4]. Seminal fluid and transvaginal collected oocytes are potential sources of microbial contamination of an IVF-ET culture system [5]. Kastrop *et al*. [4] reported 32% embryo contamination is owing to bacteria in semen.

Bacteriospermia is commonly in semen analysis. The prevalence of bacteriospermia in both men of proven fertility and of subfertile relationships ranges from 10% to 100% [6]. Certain bacteria such as *Staphylococcus* epidermidis can be identified in healthy reproductive men [7]; meanwhile, the most common isolated pathogenic bacteria are *Escherichia Coli*, *Chlamydia trachomatis*, *Ureaplasmaurealyticum* [7,8], which attributed to systemic and local reproductive tract infections, or to contamination post-ejaculation [9]. It was reported that the incidence of infection after intra-uterus-insemination (IUI) of husband semen varied from 1.83‰ to 2.1‰ [10]. Therefore, effective semen preparation protocols should be used to remove bacteria from semen [11], particularly the most harmful bacteria.

Bacterial presence in semen is mostly attributable to contamination by skin flora [12,13], the WHO only gives the general recommendations of strict hygiene and passage of urine before producing semen sample by masturbation [14]. A standard of hygiene procedures can avoid infection of staff and patients and contamination of the culture dishes [15].

Several interventions have been used to eliminate microorganisms from semen. Antibiotics are added to semen extenders to control the growth of bacteria contaminating semen during collection, but they may adversely affect the growth rate of pre-implantation embryos [16]. Furthermore, the addition of antibiotics may contribute towards the development of antibiotic resistance [17]. Moreover, genes for antimicrobial resistance are readily exchanged between reservoirs in humans, farm animals, and companion animals [18]. For these reasons, prudent use of antibiotics is advocated; i.e., antibiotics should only be used when strictly necessary, for therapeutic purposes, and after testing for bacterial sensitivity to the proposed therapeutic agent [19].

Another alternative to adding antibiotics to semen extenders may be to remove bacteria from semen by physical means [19]. Previous studies have shown that bacterial contamination in boar semen could be removed or reduced by Single Layer Centrifugation [20]. Similar results were reported in human semen preparations [21]. Additionally, one study found that semen preparation by swim-up in an antibiotic rich culture medium removed 95% of organisms [22]. However, the concentration of bacterial colonies, one of the key factors in the development of infection, is rarely discussed. Furthermore, no studies have been carried out to evaluate the efficiency of combined procedures for sperm preparation to eliminate bacteria from the final sperm media. At our reproductive center, one couple experienced recurrent embryo contamination, and both cycles were confirmed as *E. coli* infection from semen. Fortunately, at the second cycle, only some of the culture droplets were contaminated, and after transferring two embryos from contamination-free culture droplets, two healthy babies were delivered at 38 weeks.

Considering the bacteria originated in the semen, we compared the differences between the two-cycles, especially regarding semen preparation. We found that a combination of discontinuous density gradient centrifugation and swim-up was used in the second cycle, whereas only discontinuous density gradient centrifugation was used in the first cycle. Therefore, we speculated that different semen processing methods may have different success rates in removing bacteria. In this study, the efficiency of bacterial clearance was evaluated between density gradient centrifugation, swim-up, and a combination of discontinuous density gradient centrifugation and swim-up.

2. Materials and methods

2.1 Sperm source

The semen samples were collected in a sterile container from 46 men who were undergoing routine semen evaluation. Before submitting a sample, participants were required to abstain from ejaculation for 4 or more days and wash their hands immediately before collection. According to WHO guidelines [14] for semen testing, men who had received antibiotic treatment within the past three months, had a semen volume <2 mL or white blood cell counts >1 million/mL or ≥ 5/HPF on microscopic examination were excluded.

2.2 Preparing the semen by different method

Each semen sample was split into four parts; 0.5 mL aliquots were withdrawn from each sample and processed (Fig. 1).

In O-group, no semen pretreatment was conducted, the original semen sample was inoculated directedly. As the bacterial colony count was too high to be calculated in preliminary experiments, we diluted 1:64 with saline and took 0.5 mL of diluted semen for bacterial culture in D-group, the density gradient was prepared in a sterile 15 mL conical centrifuge tube by layering 0.5 mL of 45% (v/v) medium over 0.5 mL of 90% (v/v) medium (Spermgrad, Vitrolife, Sweden). 0.5 mL liquified ejaculate was carefully placed on top of the upper gradient phase and the sample was centrifuged at 300 g for 20 min. The supernatant was removed, and the pellet was resuspended in G-IVF plus (Vitrolife) by gentle pipetting and was re-centrifuged at 300 g for 10 min. The supernatant was discarded, and the final pellet was resuspended in a 0.5 mL of G-IVF plus medium for microorganism culture.

For S-group, we placed 0.5 mL of semen in a sterile 15 mL conical centrifuge tube and layered 1.0 mL of G-IVF plus over it. The tube was inclined at an angle of approximately 45° and incubated for 1 h in a 5% CO_2 atmosphere at 37 °C. The upper 0.5 mL was collected for use in the microorganism culture.

In the D+S group, after density gradient centrifugation as described above, the resuspended pallet with layered under 1.0 mL of G-IVF plus and an identical swim-up protocol was performed as for S-group.

2.3 Bacterial culture and analysis

A 0.5 mL sample from each group was inoculated on to duplicate Columbia agar plates with 5% horse blood (Biomerieux, Craponne, France) and incubated under 5% CO_2 atmosphere at 35 °C. Each plate was examined daily for 3 days, and discrete colonies were counted each day. All isolates were identified by the VITEK-2 system (Biomerieux, Craponne, France).

2.4 Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS V 19.0, IBM Statistics, Armonk, NY, USA) for Windows. The figures were plotted using R language. Because of the non-independence of the samples, clearance rates for the three procedures were compared using the marginal homogeneity test and McNemar's test. Comparisons of bacterial colony counts between the four groups were performed using the Friedman test. A Bonferroni correction was used to account for the six simultaneous pairwise comparisons. A level of $p < 0.01$, was considered statistically significant.

3. Results

Bacterial clearance was evaluated using three different levels: first, samples were evaluated for the presence or absence of bacteria after the procedure; second, the reduction in specific bacterial strains was evaluated; and third, the bacterial colony counts after the procedure were evaluated.

3.1 The bacterial clearance rates

Initially only 8.7% (4/46) of samples contained no bacteria. After the three different processes, the bacterial

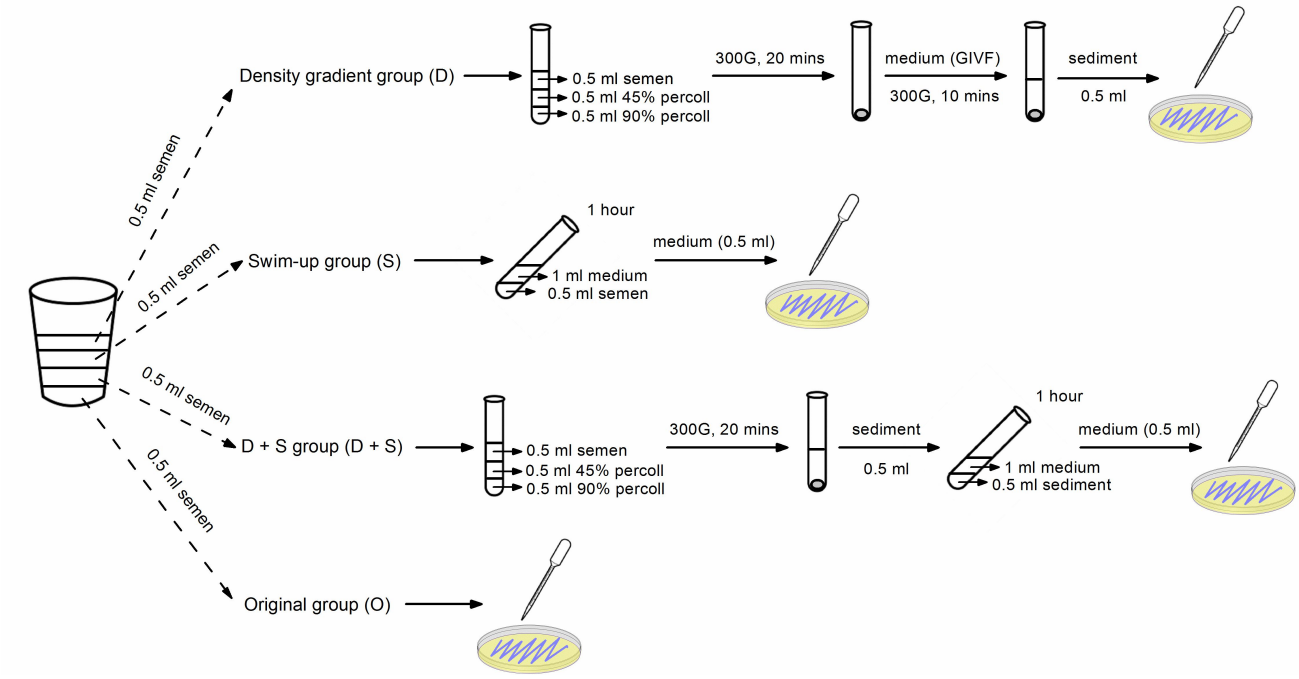

Fig. 1. Workflow diagram. Diagrammatic semen preparation protocols.

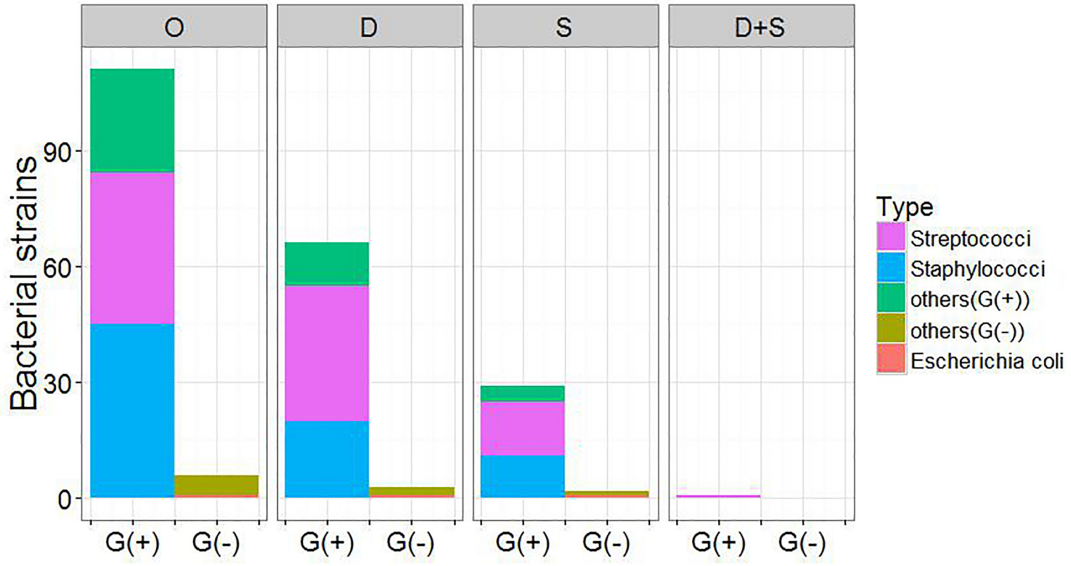

Fig. 2. The number and type of gram-positive bacteria strains (include G (+)) and gram-negative bacteria strains (G (-)) in each group.

clearance rates were 23.8% (10/42) for D-group, 57.1% (24/42) for S-group, and 97.6% (41/42) for D+S-group. These differences were statistically significant ($p < 0.001$).

3.2 Distribution of bacterial strains

Multiple bacterial strains were observed in 37 out of 42 samples, with a total of 117 different bacterial strains in all samples of the original semen. Gram-positive bacteria represented the majority of the strains of bacterial contamination. Among them, 38.5% were *staphylococci* and 33.3% were *streptococci*. Whereas gram-negative bacteria in the semen samples accounted for only 5%. Following density gradient centrifugation in D-Group, 44.4% of the *staphylococci* and 89.7% of *streptococci* were not eliminated. The corresponding rates were 24.4% and 35.9% in S-group, and there were no remaining strains of *staphylococci* and only 2.6% of in D+S-group (Fig. 2).

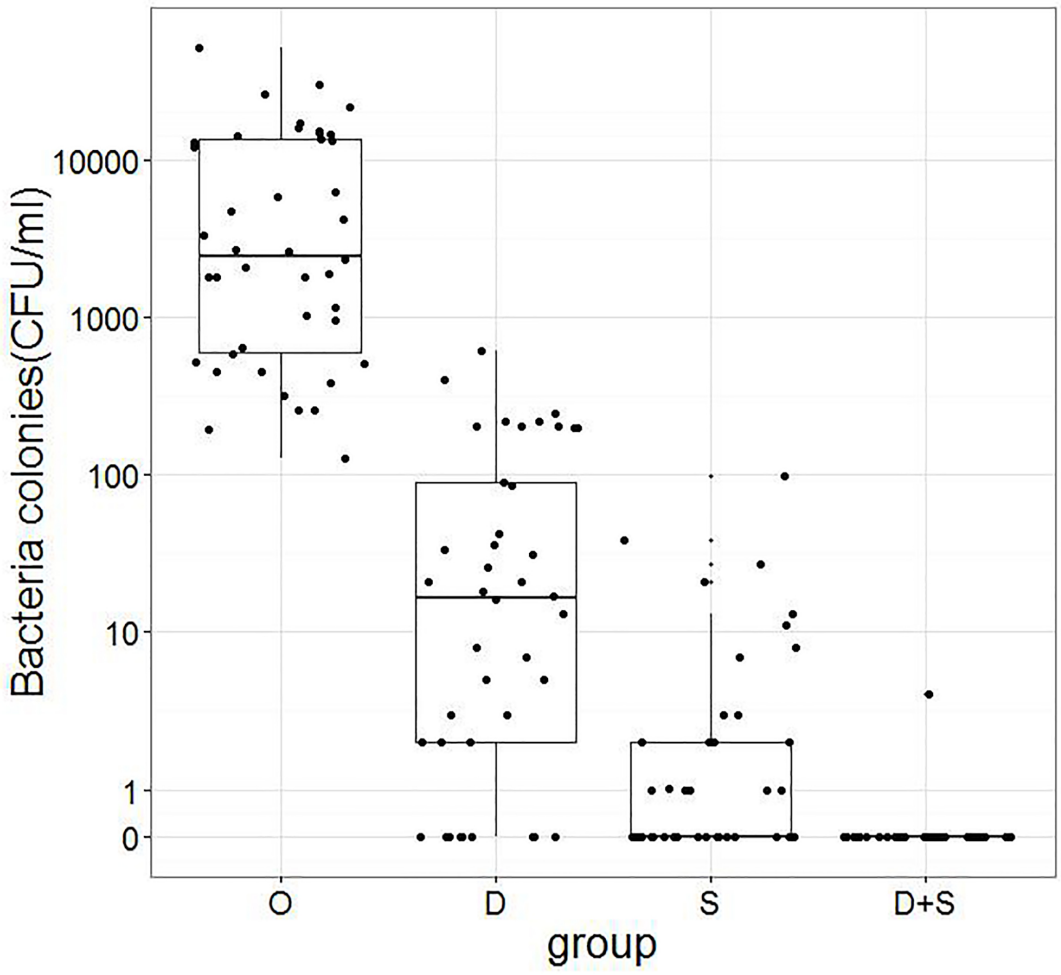

Fig. 3. Boxplot of bacterial colonies in original group (O), density gradient centrifugation group (D), swim up group (S) and combined group (D+S). Dots present individual values with a value of zero for those on the X-axis. The box- and whisker-plots report the 95th, 75th, 50th, 25th, and 5th percentiles for each group.

3.3 Bacterial colonies after different process

The most significant outcome of this study was the bacterial colonies between different preparing protocols. The 42 original semen samples contained between 100 and 10,000 colony forming units (CFUs) per milliliter. The number of bacterial colonies fell dramatically after processing. In D-group, bacterial colony contaminated samples contained between 1 and 1000 CFUs/mL. 10 samples appeared to be completely sterile. In S-group contaminated samples contained between 1 and 100 CFUs/mL and half of the samples were bacteria free. In D+S-group, there was only 1 contaminated sample that contained 4 CFUs/mL (Fig. 3).

4. Discussion

Density gradient and swim-up are the most commonly used protocols for semen preparation. Sperm preparation using density gradient centrifugation usually results in a fraction of highly motile spermatozoa, giving good separation from other cell types and debris [14]. Alternatively, spermatozoa may be selected by their ability to swim out of the seminal plasma and into culture medium. These sperm preparation protocols have been developed with the aim of preparing a highly motile suspension of spermatozoa that are removed from the non-sperm components of semen such as leukocytes, bacteria, and viruses [23]. Previous literature has explored the efficiency of different sperm preparation protocols according to their bacterial removing efficiency. The bacterial removing efficiency varied from 40–70% for density gradient or by swim-up [24–27]. It was found that both of these methods could reduce the bacterial content in semen. The swim-up protocol has been found to be more efficient in clearing bacteria from the seminal fluid compared to density gradient centrifugation. The colonies can decrease from 30 colonies/10 μL to 3 colonies/10 μL after density gradient centrifugation, and when a strict aseptic laboratory procedure was used, the colonies was further decreased to 0.13 colonies [21]. Unfortunately, neither of these methods could completely remove all bacteria [21,24,25,28,29].

The results of the current study are consistent with previous findings. We found that when density gradient and swim-up were used, bacteria in semen were completely removed in 23.8% and 57.1% of samples, respectively. Remarkably, when the two procedures were combined, only one contaminated sample was found; therefore, bacteria were removed in 97.6% of samples. This indicates that the combined use of the two semen preparation protocols is highly efficient in removing bacteria. Moreover, the bacterial colonies fell dramatically to 4 CFUs/mL.

Inconsistent effects in removing bacteria from insemination samples are owing to the different mechanisms for separating sperm. The method using centrifugation of seminal plasma over density gradients consisting of colloidal silica coated with silane, separates cells by their density [14]. Morrell *et al.* [30,31] describe that bacteria have a lower capacity to multiply after passing through the colloid; however, some specific bacterial types can adhere to the sperm surface (including the flagellum) to induce sperm agglutination. Whereas for the swim-up protocol, bacteria are unable to move fast [5], they can only move through diffusion from the spermatozoa pellet into the overlaying swim-up medium. In comparison, sperm, owing to their relatively high motor speed of 30 μm/s [32] can rapidly move into the overlayed swim-up medium and thus effectively separate themselves from the bacteria. When the procedures are combined, the advantages of both methods are evident.

The other contribution of our study is to evaluate the bacterial colony after preparing, the reduction in CFUs is meaningful in clinical work. Zhu *et al.* [1] and Kastrop *et al.* [4] reported that infections are never detected after intracytoplasmic sperm injection (ICSI) and the use of ICSI can effectively prevent the occurrence of embryo contamination [4]. This could be due to the isolation of a single motile spermatozoon. They suggest that approaches that minimize CFUs as much as possible are more effective at avoiding contamination. In our study, colony count declined significantly from the original semen to density gradient centrifugation to the swim-up procedure until only a single sample with just 4 CFUs/mL was detected after the combined procedure. This could explain why the original couple had partially contaminated embryos during their second IVF cycle in our reproductive center.

In our study, *E. coli*, which was the main pathogenetic microorganism of embryo contamination, was detected in only one sample for an incidence of 2.4% with 1920 CFUs/mL in the original sample, 26 CFUs/mL after density gradient centrifugation, and 4 CFUs per mL after swim-up procedure. There were no bacterial colonies detected after the combined procedure in this sample. Using electron microscopy, Diemer *et al.* [33] investigated why the swim-up procedure might be superior to density gradient centrifugation for eliminating *E. coli* . This revealed the adhesion of *E. coli* to spermatozoa causing ultra-structural damage and probable increased immobilization. Because the swim-up procedure utilizes the mobility of sperm to purify and concentrate them, this may explain why it is superior in clearing *E. coli* from the specimen.

Regarding limitations, one weakness of this study was that there was only one case of *E. coli* contamination so no definitive conclusions can be drawn regarding the best management of *E. coli* contaminated specimens. We also noted that the number of sperm recovered following the different procedures was not captured; however, the combined procedure produced a lower sperm count and therefore may not be suitable for all cases.

5. Conclusions

In conclusion, density gradient centrifugation followed by swim up appears to be substantially more efficient than either method alone in eliminating bacteria from semen samples. Strong consideration should be given to combining the two methods when preparing sperm for IVF treatment, especially with couples who have struggled with recurrent bacterial contamination of embryos.

Author contributions

LS was responsible for the conception and design of the study; interpretation of data; revised the article critically for important intellectual content; and approved the final draft for publication. ZC, HW, LY and WQ contributed to collect and prepare the semen. LJ contributed to bacteria culturing and deification. CO responsible for Statistical Analysis. All authors contributed to drafting and revising the article for important intellectual content and approved the final manuscript.

Ethics approval and consent to participate

This trial was approved by the Guangzhou Women and Children's Medical Center research ethics committee (Approval number: 2017-04) and informed consent was obtained from all patients.

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Conflict of interest

The authors declare no conflict of interest.

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