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The Ability to Hold Sperm at Room Temperature Appears Rate-Limited by the Sample's Native Bacterial Load

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Abstract

Infertility treatment is an ever-evolving field driven by both scientific advances and patient expectations. Unlike previous generations, modern patients increasingly seek autonomy and flexibility in their care. Although standard semen analysis remains the cornerstone of male infertility assessment, the COVID-19 pandemic has amplified patient demand for greater flexibility in the timing and location of semen collection. This study explores a potential limitation of that convenience, namely the presence of bacteria within the male reproductive tract. Our preliminary findings suggest a direct correlation between intrinsic bacterial load and the ability of semen to maintain motility over extended periods at room temperature. This relationship is particularly relevant in the context of sample transport, where delays, such as those incurred during shipping to referral laboratories, may impact the accuracy of semen analysis results.

Keywords

Sperm, Bacteria, Time-Delay, Motility, Collection Device

1. Introduction

Male factor infertility represents a significant proportion of couples having difficulty achieving pregnancy, with estimates reported to range from 30% - 50% [1]. Therefore, it is imperative to include the male partner in any workup that might lead to infertility treatment. The semen analysis remains the gold standard assessment tool of male infertility. Using standards established by the World Health Organization, semen samples are assessed for counts, motility parameters, and morphology [2]. Traditional, standard sample collection practices include having

the male patient in a private room near the andrology laboratory to limit exposure of the sample to fluctuations in temperature and to control the time between the collection and analysis.

However, during the COVID-19 pandemic, many laboratories shifted their protocols to at-home collection to meet regulatory requirements, but inadvertently allowed patients some flexibility with the collection process [3]. Several previous studies have shown no significant differences in patients who collect samples at home versus in the clinic [4] [5], and some studies may even suggest increased parameters with at-home collections [6]-[8]. The WHO provides guidance to patients who may collect samples at home for exceptional circumstances, such as a lack of adequate facilities near the laboratory, but notes that the sample should be delivered within 1 hour of collection [2].

However, since COVID patients have demanded that the flexibility remain in the collection process, clinics have had to adjust this guidance to meet patient demand. One recent study showed that semen samples collected and stored in the laboratory and those collected at home and mailed to the laboratory within 5 - 6 days showed similar parameters to those collected on day 0 [9]. Further, previous work done on semen collection devices specifically has shown that semen parameters can be preserved for 24 hours while held at room temperature [10]. However, the same study demonstrates a significant variability in samples stored beyond the 24-hour time point, leading to an investigation of the sources of the variability. It is well documented that bacteria within the male ejaculate are thought to contribute to deterioration in spermatogenesis and function by changing biochemical or physicochemical properties in vivo; leukocytes within seminal fluid may also induce antimicrobial responses (*i.e.*, reactive oxygen species) that negatively impact semen quality and thus contribute to infertility. *Staphylococcus aureus*, certain virulent strains of *Escherichia coli*, and *Ureplasma urealyticum* have all been associated with lower semen density and motility [11]. It is thought that about 10% - 15% of men suffering from male infertility have acute or chronic infection of the male urogenital tract, and semen may also be contaminated during its passage from the testes to the glans and foreskin. A number of animal studies have associated decreased sperm motility with bacteriospermia [6]. The purpose of this study was to determine if there was an association between deterioration of semen sample parameters and the presence of bacterial load contaminants at the time of collection.

2. Materials and Methods

This pilot study examined the association between bacterial presence and the maintenance of semen motility parameters over 96 hours. Seventeen semen samples were obtained from patients undergoing fertility workup following routine semen analysis under an IRB-exempt protocol. Inclusion criteria required a sample volume of at least 2 mL, a sperm concentration of at least 20 million per mL, and an initial motility greater than 20%. After clinical analysis, samples were deidentified

in accordance with IRB guidelines and transferred to the research team. Each sample underwent repeat semen analysis using a computer-assisted semen analyzer (CASA; IVOS II, Hamilton Thorne, Beverly, MA). Bacterial load at the time of collection was estimated and classified as none, mild, moderate, or heavy. Two smear slides were prepared for subsequent Gram staining. Samples were then divided between devices designed for semen collection (DISC, tradename ProteX, RSI, Frisco, TX), which have been shown to maintain motility for extended periods [12] [13]. To support room-temperature storage, samples were supplemented with one of two media: a standard sperm medium (SSM; Irvine Multipurpose Handling Media, Fujifilm-Irvine, Santa Ana, CA) or a proprietary medium (PM) formulated for room-temperature cell storage. For storage preparation, 1 mL of the assigned medium was placed into the DISC, followed by the addition of the semen sample and a 10-minute equilibration period. An additional 4 mL of fresh medium was then added, and samples were stored at room temperature for up to 96 hours. Semen analysis and Gram stain slide preparation were repeated at 24-hour intervals until 96 hours or until motility reached zero. Gram staining was performed using a standard clinical kit (Thermo Scientific Remel Gram Stain Kit, Thermo Fisher, Waltham, MA) according to the manufacturer's protocol. Bacteria were classified as Gram-positive, Gram-negative, or present, and further categorized by abundance as none, mild, moderate, or heavy based on expert evaluation. Motility data showed a strong correlation in decline over time regardless of media type ($R^2 = 0.992$); therefore, data from both media groups were combined for subsequent analyses. Motility values were normalized to time zero, and samples were grouped by bacterial load based on Gram stain results. Statistical analysis was performed using ANOVA, with within-time comparisons conducted using Tukey's mean separation test.

3. Results

Although the CASA system recorded multiple parameters of sperm movement, the primary outcome assessed was the maintenance of sample motility during extended *ex vivo* storage at room temperature. Motility measurements were highly correlated within samples regardless of media type ($R^2 = 0.992$). Therefore, data from the PM and SSM at each time point were treated as repeated measures rather than independent variables. Of the 17 samples, three were found to have no visual signs of contamination, ten had moderate levels of bacteria present, and four had heavy bacterial loads at zero hours. Initial motility varied between 20% and 86%, and only a single sample with a suspected heavy bacterial load at collection demonstrated a significant loss of motility parameters in the first 24 hours compared to the initial motility ($P < 0.007$). Based upon visual assessment of bacterial load, over half ($N = 8$) of the samples with a suspected moderate to heavy bacterial load demonstrated decreased motility at 48 hours (**Figure 1**; $P < 0.007$). An additional three samples with moderate bacterial loads demonstrated decreased motility at 72 hours ($P < 0.001$). However, four samples, including the three samples initially

to have no bacterial load, and one sample assessed initially as having a minimal suspected bacterial load, demonstrated no statistical decrease in motility at 96 hours (P = 0.820).

These findings correlated well with Gram stain results (R^2 = 0.983). While no samples were found to be entirely devoid of bacterial presence after Gram staining, they were easily categorized as having mild, moderate, or heavy bacterial loads at the time of collection using stained slides and the assessment of bacterial load. The three samples deemed to have no bacteria and the one sample deemed to have only a minimal bacterial load by initial visual assessment (N = 4) comprised a group deemed to have mild bacterial contamination by Gram stain (Positive or Negative). The four samples thought to be heavily contaminated by initial visual observation were confirmed by Gram stain, and the remaining nine were classified as having a moderate bacterial load.

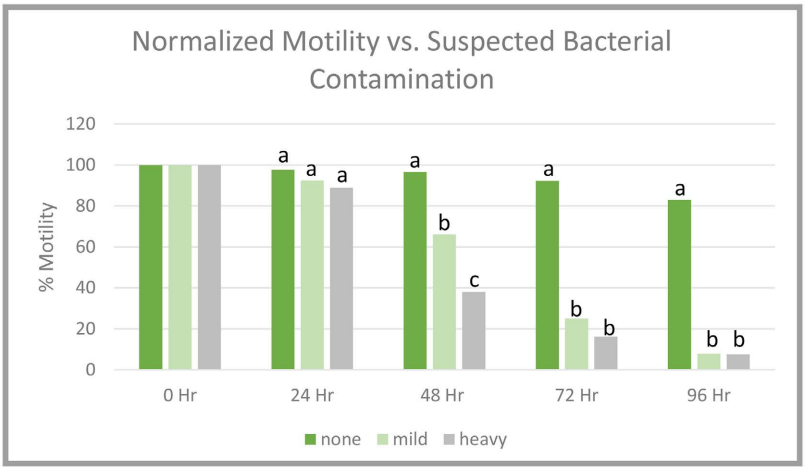

Figure 1. Semen samples with what appears to be bacterial contamination at collection exhibit decreased motility over a 96-hour extended incubation time at room temperature (P < 0.001).

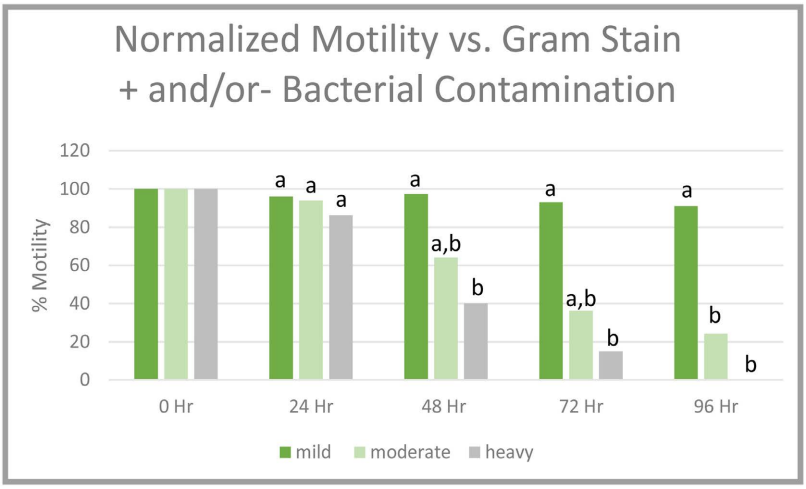

Figure 2. Semen samples with any form of bacterial contamination at collection, as detected by Gram stain, exhibit decreased motility over a 96-hour extended incubation time at room temperature (P < 0.001).

As with the visual assessment, motility decreases faster with a heavier initial bacterial load regardless of cells staining Gram positive or negative (Figure 2; $P < 0.001$). Further, the same pattern was seen when comparing motility to either Gram-positive (Figure 3; $P < 0.001$) or Gram-negative load (Figure 4; $P < 0.001$). With a single exception, none of the samples appeared to increase in bacterial load over the 96-hour incubation period as detected by either gross observation or during the Gram stain evaluation, suggesting the decrease in motility might be due either to a toxin from the bacteria or bacterial exhaustion of media nutrients rather than direct bacteria to sperm cell interactions.

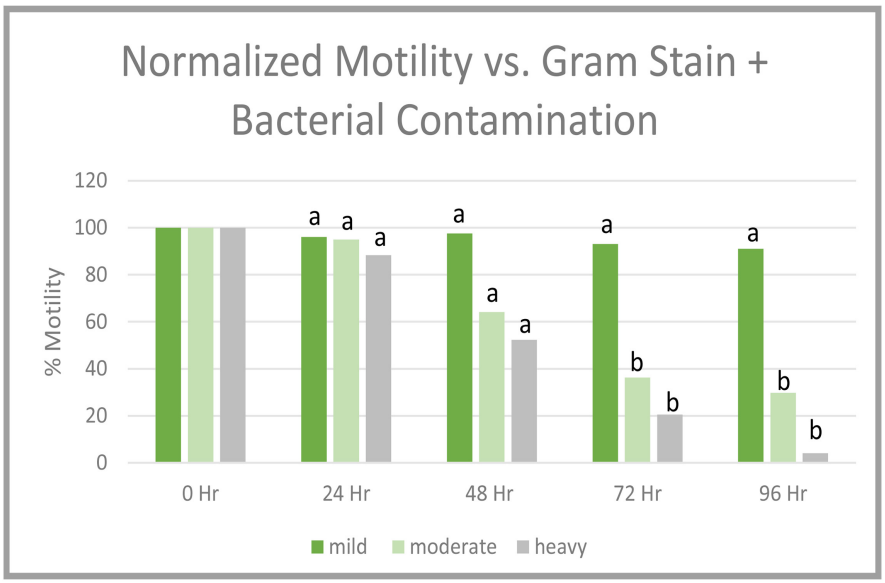

Figure 3. Semen samples with Gram-positive bacterial contamination at collection exhibit decreased motility over a 96-hour extended incubation time at room temperature ($P < 0.001$).

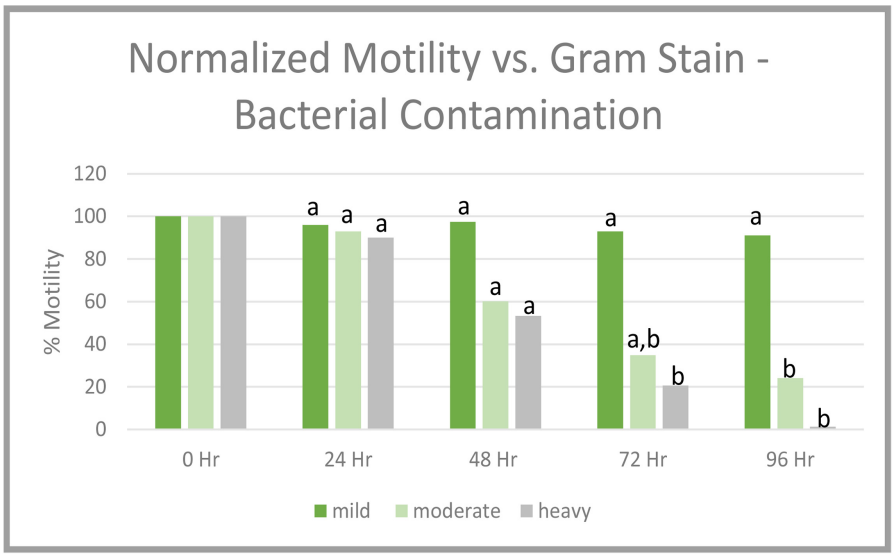

Figure 4. Semen samples with Gram-negative bacterial contamination at collection exhibit decreased motility over a 96-hour extended incubation time at room temperature ($P < 0.001$).

4. Discussion

The standard of analyzing semen as part of the workup for male factor infertility or infertility treatment has traditionally involved collecting and processing samples within an hour, with some rare exceptions. Therefore, fertility centers historically insisted that these samples be collected on-site for immediate processing [14]. Yet numerous studies have described both perceived and functional psychological stressors, which may decrease the quality of the sample or prevent its collection altogether [15]-[17]. Diagnostically, such issues might present a false assessment of what treatments are required to achieve pregnancy, adding more stress and expense to a couple's fertility journey. Further, the lack of semen at critical moments in the fertility treatment cycle is obviously limiting any chance of treatment success.

Government restrictions put in place during the global COVID-19 pandemic forced most clinics to adopt the process of at-home collection into their practices in order to continue operation. Studies reviewing semen quality found no detrimental effects on either semen parameters or clinical outcomes due to the expanded window between collection and processing [13] [18]. Since the pandemic, patients continued to desire the convenience of at-home collection processes, which led to a loosening of clinic policies and the creation of a new industry of mail-in analysis and cryopreservation [18] [19]. While there are studies suggesting little to no difference in a mail-in procedure and in-clinic collection, others suggest a deterioration in semen quality due to the length of time between collection and processing.

Previous studies leave little doubt that the presence of bacteria in the semen is detrimental to semen quality [20]-[22]. Based upon previous casual observation, the objective of the present study was to determine the role, if any, of bacteria as a rate-limiting factor in extended sperm storage at room temperature, approximating the conditions associated with semen transport. It is well documented that semen kinetic parameters decrease over time. However, it remains unclear why some samples deteriorate much faster, even when overall semen parameters such as volume, count, and motility are very similar. In the present study, bacterial load was examined for its association with decreased semen parameters and whether it might therefore decrease the usefulness of semen for both diagnostic and treatment purposes. While the authors consider this a pilot study limited by the number of samples included, the data suggest that the presence of more than a mild bacterial load caused a decrease in observed semen parameters in semen stored for more than 24 hours at room temperature, and might make all samples suspect for use without considering bacterial issues. Interestingly, with only one exception, bacterial load did not appear to increase during storage, suggesting antibacterial measures in media are doing an adequate job of inhibiting bacterial growth. However, the data show a clear association between the bacterial load present and decreased motility. This suggests the cause is related to toxic compounds or metabolites released by the bacteria or the ability of the bacteria to limit access to

nutrients needed for motility.

Additional studies need to be undertaken to determine how long fresh, extended human semen samples can be held outside the body, to provide accurate and standardized results for diagnostic purposes, and whether additional preparation could improve or lengthen the technique. It is recognized that the current study is not only limited by sample size, but also by the parameters measured. It was also limited by not knowing demographics about the donors (profession, social habits, and other habits that might influence bacterial load). Future, better-controlled studies are needed to determine the usefulness of semen extended at room temperature beyond 24 hours for diagnostic purposes. Data for this and other studies would discourage any metabolically active sperm from being stored more than a few hours ex vivo in treatment procedures.

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Conflicts of Interest

Both SP and LP would like to acknowledge a potential conflict of interest as they are inventors of the ProteX technology and stockholders in RSI. SP is also a paid consultant.

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