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Can Westgard Quality Control Rules Determine the Suitability of Frozen Sperm Pellets as a Control Material for Computer Assisted Semen Analyzers?

[Male Infertility]

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Outline

Graphics

Abstract

Purpose: To evaluate the drop-to-drop and pellet-to-pellet repeatability and stability of frozen sperm pellets.

Methods: Ten pellets were thawed per batch (low and normal concentration) and evaluated by two investigators to establish a quality control chart. Then low and normal concentration pellets were thawed and evaluated daily for 10 days by both investigators. The values for both investigators were averaged and plotted on the chart.

Results: The low sperm concentration specimen had a systematic error while the normal sperm concentration specimen had a random error as well as a systematic error. The low sperm concentration specimen violated the warning rule for motility whereas the normal concentration violated the warning rule, the random error rule, and the systematic error rule when applied to motility.

Conclusions: Frozen sperm pellets are not acceptable as a daily-use quality control material for semen analysis when using a computer assisted semen analyzer.

INTRODUCTION

The issue of quality control of semen analyses has long been a thorny one for scientists working in clinical Andrology laboratories. One of the major stumbling blocks of this issue is the imprecision of the analytical method of semen analysis. All analytical methods require the performance of quality control procedures to verify the proper functioning of the method. These control procedures require long-term, repeated analysis of control materials followed by the application of statistical procedures that will aid in the characterization of the error of the analytical method.

Imprecision of an analytical semen analysis method can be divided into four areas: 1) the instrumentation used to evaluate the semen specimen, 2) the counting chamber used, 3) the differences in techniques used by individual laboratorians, and 4) the analyte.

In recent years, the development of an instrument called the computer assisted semen analyzer (CASA) has greatly improved the standardization of semen analysis (1,2). The CASA is primarily computer-driven and operator intervention is required only in selecting fields for analysis and reviewing results. The algorithms used to determine sperm concentration and motility characteristics are programmed into the software and cannot be changed by the operator. Analysis parameter settings (either preset by the manufacturer or developed by the operator) do not change from analysis to analysis. In addition, no external reagents are needed and no calibration of the CASA by the operator is required.

The MicroCell disposable sperm counting chamber (Conception Technologies, San Diego, CA), with its fixed cover slip and standardized chamber depth, has greatly reduced counting chamber-related error (2,3). The chamber fills by capillary action and cannot be overfilled. Under filled chambers and chambers demonstrating air bubbles are readily observable and easily discarded. The CASA is compatible with MicroCells and, when they are selected in the CASA's software as the chamber to be used, calculations of sperm concentration and motility are based on the MicroCell's chamber depth of 20 μm.

Error can be introduced into an analytical method by the specimen handling techniques of the laboratorians. Failure to adequately mix a specimen prior to sampling, dilution errors, pipetting errors, and incorrect loading of counting chambers may all contribute error to a method. Proper training and periodic review of the work habits

of all andrology laboratory personnel will help ensure that semen analyses are performed correctly and in the same manner by all laboratorians. This similarity in performance was confirmed when we reported no differences between two investigators collecting semen analysis data (2).

Characteristics of the analyte in question also may contribute error to a method, particularly if the analyte is semen. Because semen is a nonhomogenous fluid, it is difficult to obtain an even distribution of sperm cells in seminal plasma (4). In addition, the motility of sperm decreases over time (5). Furthermore, over time, sperm cells clump to each other and to debris in the seminal plasma (4). Because of the aforementioned instability of semen, its suitability as a control material for semen analysis is being brought into question.

Characteristics of a good control material include 1) similarity to "real" patient specimens, 2) availability in large enough quantities to allow evaluation over a long period of time, 3) availability in concentrations representative of normal values or important medical decision-making levels, 4) similarity in concentration from drop-to-drop and vial-to-vial (precision), and 5) stability over a long period of use (a year or more) (6).

Semen Concentration and Motility Control (CMC) (Conception Technologies, San Diego, CA) is marketed as a control material for the CASA. Semen CMC is a pelletized frozen human semen product of known sperm concentration and motility. It is available in an abnormal or "Low" concentration and motility and a "Normal" concentration and motility to provide a two-level control material. This product meets the first three requirements of a good control material described above. Whether it meets the last two requirements needs investigation.

The purpose of this study is to investigate whether, when used with a stable analytical method, Semen CMC meets the fourth and fifth requirements of a good control material, i.e., similarity in concentration from drop-to-drop and pellet-to-pellet and stability over a long period of use. We also want to discover if Westgard Quality Control Rules can be used, in this instance, to determine the stability of a control material rather than the stability of an analytical method. Also at issue is whether a control material of questionable stability may still be useful in a laboratory quality control program.

MATERIALS AND METHODS

Parameter Settings for CASA

The CASA used for this study was the Hamilton-Thorne Internal Visual Optics (IVOS), Version 10.7b (Hamilton-Thorne Research, Beverly, MA). The parameter settings used for the analysis of Semen CMC in our laboratory were as follows: frames acquired: 7; frame rate: 60 Hz; minimum contrast: 30; minimum size: 2 pixels; static head size gates: 0.53–2.99; static head intensity gates: 0.45–1.23; nonmotile head size: 5 pixels; nonmotile head intensity: 100; medium path velocity (VAP) cutoff: 25.0 μm/s; low VAP cutoff: 5.0 μm/s; slow cells motile: yes; and threshold straightness: 80.

The IVOS is equipped with a built-in "Playback" feature that allows the operator to monitor how well the computer is "capturing" the sperm analyzed in a particular field. Motile sperm are labeled with green tracks indicating how far they moved in the field during the 0.67 s they were analyzed by the computer. Nonmotile sperm are labeled with red dots. This labeling allows the operator to visually assess whether the computer has failed to capture sperm in the field or if debris commonly found in semen specimens has been erroneously labeled as sperm. Adjustable gates for sperm head area (μm^2), image intensity, and sperm head elongation allow the operator to change these settings to give the best labeling of sperm and screen out mislabeled debris.

Definition and Application of Westgard Quality Control Rules

In 1981, Westgard and coworkers published a series of control rules that are now commonly referred to as "Westgard Rules" (6). When used in combination with a Shewhart quality control chart (an *X* versus *Y* graph

demonstrating the mean and standard deviation [SD] of repeated measurements of a control product; Figs. 1 and 2), Westgard Rules provide a simple statistical procedure to determine whether observed control measurements represent the stable or unstable performance of an analytical method. When used with a *stable* control material that is analyzed repeatedly over a long period of time, Westgard Rules can tell an investigator when an analysis method is "out of control" and thus, that any subsequent patient data should not be used by the physician in making diagnostic decisions.

Graphic
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Fig. 1. Shewhart quality control charts plotted with Low and Normal Semen CMC sperm concentration data.

Graphic
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Fig. 2. Shewhart quality control charts plotted with Low and Normal Semen CMC sperm motility data.

Six quality control rules that can be used when evaluating data are listed in Table I. A minimum of two control rules should be used, one that detects random analytical error (error that occurs on both sides of the mean) and one that detects systematic error (error that occurs on only one side of the mean). This way, the control rule violated will give some indication of the type of error occurring and will aid in problem solving (or trouble-shooting). Simultaneous use of several rules can improve the performance of a control procedure and minimize false rejection of test results. The rules can be applied "within" a control material if only one level of control is run, or "across" control materials if two levels of control are run.

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Table I. Definitions of Westgard Quality Control Rules

An algorithm for the order of application of the Westgard Rules "across" two control levels is shown in Fig. 3. In practice, control materials are run at specified intervals (i.e., daily, at every shift change, with every batch of patient specimens) and the results graphed on a Shewhart quality control chart. In this way, a large volume of data is readily available for inspection and the application of control rules can be easily accomplished.

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Fig. 3. Algorithm demonstrating the application of Westgard Quality Control Rules across control materials.

This simple statistical control procedure lends itself well to the control of Clinical Chemistry methods where, for example, the analyte being tested is glucose and a supply of a stable glucose control material is readily available. However, in our case, we have a very stable method (the CASA plus the 20 μm MicroCell and investigators that do not differ), but the stability of the control material (Semen CMC) is in question and this is what we are investigating.

Sperm Pellet Manufacturing and Determination of Pellet Mean Concentration and Motility

According to the manufacturer of Semen CMC, semen specimens provided by donors were pooled and mixed with Test Yolk Buffered Freezing medium at a ratio of 1:1. The mixture was cooled in a refrigerator for 1 h

before being frozen on dry ice as 50 µL pellets. The pellets were then stored on aluminum canes in liquid nitrogen (7).

Mean sperm concentrations and motilities for each batch of pellets was determined by the manufacturer using a replication study. Ten pellets from each batch were thawed. Three fields per pellet were analyzed on MicroCell counting chambers using a Hamilton-Thorne CASA. The same technician analyzed all 10 pellets per batch. The manufacturer provided the mean and SD concentrations and motilities to the purchaser when the pellets were shipped. This product was labeled as Semen CMC and was shipped to customers in storage canes with 50 pellets per cane.

Loading the Counting Chamber and Analyzing Semen CMC

One pellet of either Low or Normal concentration and motility was removed from the cane and transferred into an aluminum thawing block (supplied by Conception Technologies). The pellet was allowed to thaw at room temperature (approximately 23°C) for 10 min. Each pellet provided approximately 25 µL of usable product. To eliminate lot-to-lot variation, a single lot of each of the Low and Normal Semen CMC pellets was used throughout this experiment.

Two investigators alternated thawing the pellets, loading, and analyzing the chambers. Here one side of a two-chambered 20 µm MicroCell slide is defined as a chamber. Once a pellet was thawed and then mixed by repeated up and down pipetting, Investigator 1 loaded 6 µL of the thawed, mixed semen into the first chamber. This chamber was then analyzed on the CASA. When this first analysis was finished, Investigator 2 performed a second analysis on the same chamber. Investigator 1 then loaded and analyzed the second chamber, which in turn was analyzed again by Investigator 2. Both of the counting chambers were loaded from the one pellet. On the second round of analyses, Investigator 2 took the lead by thawing the pellet, loading the first chamber and performing the first analysis. After the analysis of each chamber, the investigators used the CASA's Playback feature to check for proper labeling of sperm and adjusted the gates as needed.

If a chamber demonstrated air bubbles under the coverslip or failed to fill completely, it was discarded and a new chamber was loaded. The chambers were analyzed between the CASA stage positions of 7.5 mm and 0.0 mm (2). Six to seven fields and a minimum of 200 sperm cells were analyzed on each chamber (8).

The motility of thawed sperm is often slow. Therefore, the CASA was configured to count slow cells as motile (as described in the parameter settings listed above). This provided a motility threshold low enough to assure that these sperm would be captured and analyzed by the computer.

A total of 10 low-level pellets and 10 normal-level pellets were thawed and analyzed by two investigators using a 20µm MicroCell counting chamber. We had previously determined that the two investigators were not different (2), and continued to confirm this on a quarterly basis for the length of time the two investigators worked together. Therefore, we pooled the data to provide a total of 20 analyses performed for each control level. The analysis of each pellet was completed within 20 min postthaw as recommended by Conception Technologies.

Using the 20 counts described above (10 pellets ×2 investigators), a Shewhart chart was constructed for the low sperm concentration/motility (Figs. 1 and 2). Once the chart was drawn, low sperm concentration/motility pellets were thawed and evaluated daily for 10 days by both investigators, the results averaged and then plotted on the chart. A similar process was repeated for the normal sperm concentration/motility pellets (Figs. 1 and 2).

Statistical Methods

The mean, SD, and coefficient of variation (CV) were calculated for the low sperm concentration and motility pellets and the normal sperm concentration and motility pellets.

RESULTS

Table II demonstrates the mean ± SD, the acceptable ranges for sperm concentration and motility measurements (mean ± 2SD) and the CVs for 10 counts each of the Low and Normal Semen CMC pellets by two investigators.

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Table II. Descriptive Statistics of 10 Low and 10 Normal Semen CMC Sperm Pellets Analyzed by Two Investigators

Sperm Concentration

Application of Westgard Rules within the control materials demonstrated three rule violations (Fig. 1). For Low Concentration, Count Number 6 violated the 4_{1s} rule (systematic error). For Normal Concentration, Count Number 3 violated the 1_{3s} rule (random error) and Count Number 20 violated the 10_{Mean} rule (systematic error). No additional violations were found when the rules were applied across the control materials.

Sperm Motility

Application of Westgard Rules within the control materials demonstrated four rule violations (Fig. 2). For Low Motility, the result obtained on Day 1 violated the 1_{2s} rule (the warning rule). For Normal Motility, Count Number 10 violated the 1_{3s} rule (random error), Count Number 17 violated 1_{2s} rule (the warning rule), and Count Number 18 violated the 2_{2s} rule (systematic error). No additional violations were found when the rules were applied across the control materials.

DISCUSSION

Four factors play a role in determining the reliability of the analytical method chosen for semen analysis. They are 1) instrumentation, 2) counting chamber, 3) investigator variability, and 4) stability of control materials.

Instruments used for specimen analysis vary depending on the method chosen and the analyte in question. It seems only logical to assume that instruments requiring the use of many reagents and whose calibration procedures must be repeated frequently are prone to introduce more error into a method than instruments without these requirements.

In our laboratory, the CASA has proven to be a very stable instrument. For use in daily CASA quality control, we store digitized images of a low and a normal sperm concentration specimen on the computer's hard drive. These images are retrieved and analyzed each day using a constant set of analysis parameters. The CASA is so repeatable that the results are virtually identical each day.

Used with the 20 µm MicroCell chamber, the CASA demonstrates a high degree of accuracy and precision in measurements of sperm concentration and motility (2). We previously demonstrated that the MicroCell chamber is superior to either the hemacytometer or the Makler chamber for measurements of sperm concentration and motility (2).

Concurrent manual and CASA counts of the two levels of Semen CMC were not performed in this study.

Previous studies performed in our laboratory demonstrated a high degree of correlation between manually and CASA-analyzed MicroCell chambers (5). This continues to be true in ongoing intralaboratory proficiency testing (PT) events in which lab personnel perform comparative manual and CASA measurements of fresh sperm concentration and motility.

Investigator variability can introduce large errors into an analysis process. In the performance of a semen analysis, investigator errors may include mixing errors, dilution errors, chamber filling errors, and other subtle differences that may contribute to method error. Previous studies (2) and ongoing intra-laboratory PT events (comparative measurements of fresh sperm concentration and motility performed by all laboratory personnel) have demonstrated no differences between the two investigators participating in the data collection phase of this study. Therefore, we feel this part of the analysis process is also stable.

A number of different products have been offered or suggested as suitable control materials for CASA semen analyses. One product, glass calibration slides (MicroCell-CS Calibration Standard, Conception Technologies, San Diego, CA), are etched with known concentrations of "impressions." A second product is aqueous solutions of latex beads of known concentration (AccuBeads, Hamilton-Thorne Research, Beverly, MA) and similar in size to sperm heads. Both of these products provide some measure of quality control for the CASA, but neither simulates actual semen specimens nor allows for the evaluation of motility.

At the time data were being collected for this study, Semen CMC was the only commercially available semen-based product that demonstrated some of the characteristics of a good control material. That is why it was chosen for evaluation. Since that time, due to the increasing awareness of the need for quality control procedures and products for semen analysis, other products, including videotapes and other semen-based control products have become available. Initial evaluation of any potential quality control product is required to determine the product's stability. If proven stable via repeated analysis, either product of the type mentioned above could be integrated into a laboratories quality control program and Westgard Rules could easily be used to monitor method performance.

Semen CMC would seem to be an ideal control material as it is produced from pooled human semen in lots large enough to allow repeated analysis over a long period of time. Also, it is available in two concentrations to provide a two-level control material. But the fact remains that semen is a biological product and it may not be stable enough to qualify for use as a control material. Because we felt that the CASA, the counting chamber, and the two investigators were stable aspects of the semen analysis method in our laboratory, we reasoned that error seen in the method would be due to the control material itself. We investigated the stability of Semen CMC by analysis of repeated measurements of sperm concentration and motility and by the application of Westgard quality control rules to those repeated measurements.

When Westgard Rules were applied to the 20 sperm concentration measurements of the Low and Normal Semen CMC pellets, three rule violations occurred, all of which would have led to the rejection of the analysis run. No patient results could have been reported until the problem was identified and corrected and the control materials rerun and results found to fall within acceptable ranges. Two of the three violations indicated that systematic error was occurring. The third indicated random error.

Application of Westgard Rules to the 20 sperm motility measurements of the Low and Normal Semen CMC pellets demonstrated four rule violations. In this instance, two of the violations would have led to rejection of the run. The first violation indicated the occurrence of systematic error while the second violation indicated random error. The other two violations were of the "warning" rule (1_{2s} rule) which signals a need for further inspection of the data, but not necessarily rejection of the analytical run.

Since we had already ruled out the CASA, the counting chambers and the investigators as causes of method error, the error we are detecting with Westgard Rules appears to have Semen CMC as its source. The large CVs

calculated for the Low and Normal sperm pellets as shown in Table II demonstrate a lack of stability of Semen CMC.

Repeated violations of Westgard quality control rules should cause the laboratorian to investigate the reasons for the violations. Trouble-shooting the CASA, while minimal, may include 1) checking the parameter settings, 2) checking that a dilution factor has been entered when needed, 3) checking that the correct chamber type has been selected, and 4) monitoring of comparative CASA versus manual semen counts with ongoing PT events.

If all CASA settings are correct, the next area to be investigated is the counting chamber. The investigator should check to see that 1) the chamber has been completely filled, 2) the chamber contains no air bubbles, and 3) that excessive sperm agglutination is not present in the semen specimen.

Making sure that different investigators handle specimens and perform analyses in the same manner are more difficult to control but are crucial. Investigator trouble-shooting may include 1) checking for adequate mixing of specimens prior to sampling, 2) reviewing pipetting and dilution-making techniques, 3) checking that chambers are loaded correctly, and 4) monitoring laboratorian performance with ongoing PT events.

If the instrument, chamber, and investigator are all functioning properly, the control material is the likely source of control rule violations. In this instance, the laboratory supervisor could make the decision to accept the analytical run and report patient results knowing that the control material, and not the method, is the problem.

In our hands, Semen CMC proved too unstable to use daily as a quality control product for the CASA. Perhaps improvement in the areas of cryopreservation and thawing of Semen CMC would yield a more stable, acceptable product. It could, however, be useful in laboratory PT events. Valuable information on semen analysis method performance could be obtained through comparative CASA versus manual counts of Semen CMC. Also, if analysis of Semen CMC is performed concurrently by all laboratorians, interinvestigator differences can be discovered and, with training and practice, eliminated.

In conclusion, Semen CMC proved unacceptable as a daily-use quality control material for semen analysis performed using CASA. Application of Westgard Rules to quality control data collected using a stable semen analysis method proved very helpful in our investigation of the suitability of Semen CMC as a control material. Semen CMC could prove useful in a laboratory's PT program since it allows for the measurement of sperm concentration and sperm motility. Ongoing quality control procedures are essential to ensure the medical usefulness of test results produced by the laboratory.

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