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Androlog Summary

Semen Analysis: Are 2 Counts Truly Better Than 1?

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Note: Postings to *Androlog* have been lightly edited before publication.

Sometimes questions that seem routine on their surface resonate deeply within the Andrology community.

Grace Centola writes regarding the performance of the semen analysis.

> Two labs have recently been cited by inspectors (NYS and CLIA inspectors) because sperm counts and motilities were not being done in duplicate. Apparently, the inspectors point out that a sperm count and motility must be done in duplicate, then the counts averaged, and the mean given as the value. I don't know of any lab that does the sperm count and motility in duplicate and then takes the average. Especially for busy high-volume labs, this would be very cumbersome. When using disposable slides, it becomes costly; and when using a Makler chamber, it would become time consuming as well. What is the basis for this? I know that in endocrine, no one does each serum sample in duplicate. Does anyone do counts/motilities in duplicate?

Clinical andrologists commonly assess interassay variability in the semen analysis by performing 2 or more tests at separate dates. But what of intra-assay variability?

Lars Bjorndahl references the 2002 *ESHRE Monograph.*

> In the EQC scheme of the European Society for Human Reproduction and Embryology (ESHRE) Special Interest Group in Andrology (ESHRE SIGA), the participating laboratories make duplicate counts for sperm concentration and motility, as recommended by the WHO (1999 and earlier). The reason for the counting of duplicates is to decrease the considerable risk for random errors occurring when sampling a minute volume from the semen and from the diluted sperm suspension. Another important aspect to decrease the influence of random factors is to assess sufficient numbers of sperm. If less than 400 sperm are counted, the

variation due to random factors can be greater than ±10% of the result. So the quality (and reliability) of the results from your laboratory is influenced by random factors. By counting and comparing duplicates, some errors are less likely to affect the results, and by counting sufficient numbers of cells, other random influence can be decreased. You must valuate the costs in relation to the quality of the service you want to provide.

More details on techniques for sperm counting and motility assessment (including backgrounds and theory behind recommendations) can be found in the NAFA-ESHRE *Manual on Basic Semen Analysis* (*ESHRE Monograph* 2002), also available as a pdf-file at the NAFA web site: http://www.ki.se/org/nafa/manual/manual.html.

Marc Van den Bergh replies, contrasting published recommendations to reality in practice.

The *Manual for Basic Semen Analysis, ESHRE Monograph*, June 2002, edited by U. Kvist and I. Bjorndahl, recommends on page 6, to fill the two sides of the Neubauer and to compare the counts of the two chambers. The same manual on page 10 recommends to duplicate counting for motility.

The fourth edition of the WHO laboratory manual for the examination of human semen recommends on page 14 to determinate the concentration on 2 separate preparations and on page 10 to compare the motility from 2 independent counts.

Reality is quite different for as far as I have seen it in the last 25 years and more important is the participation in external quality control programs, to estimate if you are accurate and reproducibe.

Michael Reed cites several well-established protocols for semen analysis, and also raises the question of clinical practicality.

I'd like to respond to Grace's comments on sperm counting and motility estimation, and regulatory issues. To start, I would make the assumption that the counts and motilities in the laboratories that were cited were performed using manual determinations rather than CASA. Grace, your comment on endocrine determinations is correct, in that the `moderate complexity' tests, eg, assays performed by most routine endocrine assay machines, do only require one test unit to be analyzed after control samples (also run in single units) are found to be within the working range of the assay. In the old days, with RIA, it was routine to average 2 or even 3 samples to account for technician error and the like. So in my opinion, I take this all to mean that our manual methods of sperm count and motility are considered to be more `primitive' and subject to greater error than the CASA counterparts, even though the machines use the same type of counting chambers.

And if one is using a hemocytometer (or haemocytometer) there are dilutions involved, again a technician-dependent item, and the methods for hemocytometer determinations call for `duplicates' using both sides of the counting chambers for the same preparation. I would really like to know if the interpretation by these inspectors, for the term `duplicate' was aimed at the laboratory needing to use both sides of a hemocytometer chamber, or did they actually want the laboratories to be using two hemocytometer chambers?

The Makler chamber is designed, according to the manufacturer, specifically for semen analysis, with no initial dilutions needed (although there are times when a dilution is

needed, and yes, there should be duplicate counts with dilutions). The same is true for the Cell-Vu, where the manufacturer states that a determination can be done in a single chamber. While there have been numerous papers about the variabilities between manual vs computerized analysis, and chamber to chamber variations, who has the final say over what method or equipment can be used? If the labs that were cited did not use hemocytometers but rather were using counting chambers according to the manufacturer's recommendations (eg, Makler, Cell-Vu—I can't say for sure about the other chambers) and were told to perform duplicate counts, then who is correct? The manufacturers or the inspectors?

Just to be complete, I pulled the following from the manuals that I have in the lab:

According to WHO Fourth edition, in reference to performance of manual count and motility determinations `Duplicate counts of 200 spermatozoa must be performed to achieve an acceptably low counting error,' dictating also that the haemocytometer be used. It goes on further to say that the Makler chamber is not recommended and that other methods should be validated against the haemocytometer. Determination of motility is approached in the same manner, `The count of 200 spermatozoa is repeated on a separate 10 µL specimen from the same semen sample and the percentages of each motility grade from the 2 independent counts are compared.'

According to the 2002 ESHRE *Monographs Manual on Basic Semen Analysis* (a good resource, I think), the counting chamber suggested is the Neubauer haemocytometer, and both count and motility are based on duplicate counts of at least 200 cells per count, averaging the results. There are some interesting comparisons made between the `confidence' of counts comparing the Neubauer to the Makler, in particular for semen specimens that have low counts.

In Jeyendran's 2003 *Protocols for Semen Analysis in Clinical Diagnosis*, `Determination of sperm concentration with a standard Neubauer hemocytometer procedure is detailed below. For all other counting chambers, follow the respective manufacturer's recommendations.' The directions for the hemocytometer are of course, to perform the count on both sides of the chamber, duplicates in essence. A comment by the author is interesting, to me at least, with reference to the Neubauer hemocytometer, the Micro Cell, the Cell-Vu, the Petroff Hausser Chamber, and the Makler chamber, `These various counting chambers yield at best a reliable estimate of sperm concentration.'

Obviously, we are all aiming for accuracy and precision in our quest to categorize biological variability, and even under the best conditions, we're looking at estimates. In the above-mentioned manuals, specifically the ESHRE and WHO manuals, there are sections devoted to the `confidence' achieved by counting different numbers of sperm, 200, 400, and so on. Again, CASA is more likely to be able to count these larger numbers of sperm, much more quickly and therefore achieve a higher level of confidence.

All of the rhetoric aside, if a laboratory is able to `validate' a sperm count and motility estimation procedure for any given counting chamber, manual or CASA, by repeated measurements and comparisons between `single' and `duplicate' determinations, with statistically acceptable ranges of variation, eg, the two methods are within, say, 2SD (20%), will there be, or should there be, an issue with the inspectors?

And until we all perform semen analyses in the exact same way, using the exact same

methods, and achieve acceptable proficiency testing results, aren't these `fine points' of semen analysis moot, as long as, within our individual laboratories, we are able to replicate measurements within acceptable limits, using comparisons with neat semen and control materials?

Suresh Sikka suggests the laboratory protocols be modulated by clinician and technician judgment.

(Grace) has raised some important issues and the key questions are 1) How much is too much? 2) What to sacrifice—quality or quantity and where is the compromise? 3) Who pays for the extra time, supplies, etc. involved in duplicate or multiple analyses? 4) How to approach HMOs, Medicare, etc. for upgrading the coding and billing charges for such compensations regulated by CLIA/HCFA?

Of course maintaining Q/C and Q/A in andrology labs is important. However, for routine analyses where the physicians need is to rule out male factor infertility, the judgment of laboratory personnel evaluating the semen sample should be more important, eg, for such a patient, if the initial semen sample evaluation suggests >40 million/mL sperm with >60% forward motile sperm, I don't think there is need to do a duplicate analysis so as to report an average. By counting more that 200 sperm rather than the usual 100 for motility, and counting more fields (eg, >6 fields using microcell) for sperm concentration, this should be sufficient to conclude that these parameters are normal. Similarly, for a severe oligoasthenozoospermic sample, it will be obvious that it is abnormal. It is the grey areas where the interpretations are equivocal, and in those cases, at least duplicate, and if greater than >15% CV in duplicates, then even a third observation should be made.

Susan Rothmann emphasized quality control within the individual laboratory.

Standard practice in cytometry is that, when a sample is diluted, the dilutions must be counted in duplicate. Since most lab inspectors come from classical clinical pathology, they only know about hemacytometers which must be used with a dilution. You need to make sure they understand that the Makler and most disposable chambers don't require dilution and therefore shouldn't require a duplicate count.

Most of the replies to this question assumed or recommended that a hemacytometer is used for sperm counts. Most assessments of most disposable chambers have shown them to be as good or better than a hemacytometer. We certainly have and prefer them because they don't require the duplicate counts. The Makler has not proven to be as good, which is why the WHO manual recently recommended that it not be used.

What is essential is that every lab needs to establish parameters for how many cells, fields, and chambers to count in order to create an acceptable variation. Routine quality control will help to assure that the process used actually works.

Some of the answers to Grace's question got into the issues of time and compensation for the `extra' work of counting a duplicate. If you use a hemacytometer, counting duplicates is not `extra,' rather is part of the procedure and should be factored into labor costs and then pricing.

Sally Perreault Darney notes variability in results associated with laboratory technique.

... Regarding the question of duplicate counts—I interpret this to mean duplicate

aliquots from a given raw semen sample, each diluted the same way and then loaded and counted. As such, the duplicates control for variance associated with 1) pipetting the first aliquot from a viscous sample and 2) subsequent dilution errors. However, there is a third variable that increases the overall variance, namely, variation in filling the two sides of the chamber.

The other question raised, regarding choice of chamber and automated counting (which can only be done with chambers of appropriate depth), introduces a different question. If using a different chamber gives consistently higher or lower values than the themacytometer, then should the WHO reference value be adjusted for that counting method? We recently (2003 ASA abstract by Jeffay et al) counted a single aliquot of diluted semen with the HTM-IDENT (using microcell and loading both sides with the same sample and then counting both sides) and then diluted this same preparation further and recounted it with the Neubauer hemacytometer (again both sides). Here we controlled only for variance associated with filling the chamber (not with duplicate initial aliquots of semen). We got better precision with the IVO-SIDENT (closer agreement of counts on both sides of chamber), and good correlation with the hemacytometer, but IVOS-IDENT counts were consistently lower. (Others have found the same thing). We feel that both methods are legitimate but that using the WHO reference value (20 million/mL) that was developed based on hemacytometer counts, would not be valid for our IVOS-IDENT counts. Rather, we calculated a value that would be mathematically comparable. Such a value can be important when analyzing sperm counts as dichotomous variables in order to derive risk ratios in epidemiology studies.

Christopher De Jonge also emphasizes the critical role of quality control in the individual laboratory performing semen analysis.

It is important to note that the statement in the WHO 1999 concerning the Makler and the recommendation that it not be used was later formally amended.

Susan (theory) and Sally (theory in practice) raise perhaps the most important aspects regarding counts: 1) `every lab needs to establish parameters for how many cells, fields, and chambers to count in order to create an acceptable variation. Routine quality control will help to assure that the process used actually works.' 2) `We got better precision with the IVOS-IDENT (closer agreement of counts on both sides of chamber), and good correlation with the hemacytometer, but IVOS-IDENT counts were consistently lower. We feel that both methods are legitimate but that using the WHO reference value (20 million/mL) that was developed based on hemacytometer counts, would not be valid for our IVOS-IDENT counts. Rather, we calculated a value that would be mathematically comparable.

If points #1 and #2 are used (and documented) by labs and for any chamber, then the concerns of inspectors should be made moot.

The preceding replies are but a sample of Androlog responses to Grace Centola's original post; many others sent excellent replies. The substantial volume of well-considered responses with fundamental raised questions indicates the depth of contemplation within the male reproductive community regarding its most common assay. With ever evolving laboratory technology, perhaps the first order of business in the 21st century in andrology should be to align laboratory protocols, statistical assessment, and clinical interpretation in the performance of the commonplace semen analysis.

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