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Andrology

Developing techniques for determining sperm morphology in today's andrology laboratory

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***Purpose*:** To investigate three areas: the staining of spermatozoa; the Computer Assisted Semen Analysis (CASA); and the variability of technicians.
***Methods*:** Staining experiment: sperm from 15 beef bulls were randomized to one of three staining protocols. CASA experiment: slides were evaluated using the Integrated Visual Optical System and compared to technician results. Variability of technicians: five laboratorians analyzed the same set of 20 slides six different times.
***Results*:** Staining experiment: the size of the sperm increased in proportion to increased time and heat associated with each successive protocol. CASA experiment: coefficient of variation ranged from 18.3 to 101.7% (12 slides). Variability of technicians: the mean sperm morphology results ranged from 7.3 to 15% normal forms.
***Conclusions*:** Until laboratories adhere to the universal standard set by the World Health Organization to evaluate sperm morphology, a laboratory must rely on its own quality control to insure repeatable results.

KEY WORDS: Computer-assisted semen analysis; semen analysis; sperm morphology; staining; technician variability.

INTRODUCTION

Today, one out of six couples will seek medical treatment in an attempt to conceive a child and male factor comprises 29% of the infertility in these couples (CDC, 2002). An analysis may be performed to determine if the semen from a male falls below the reference values set by the World Health Organization (WHO, 1999). Currently, these values for normal males include a minimum volume of 2.0 ml, a pH of 7.2 or greater, a concentration of 20 $\times$ 10^6 spermatozoa/mL or greater, and a total sperm number of at least 40 $\times$ 10^6 spermatozoa per ejaculate. Additional criteria are motility of 50% or more, vitality of at least 50%, white blood cell count of fewer than 1 $\times$ 10^6/mL, immunobead test with fewer than 50% motile sperm with beads bound, and an MAR test with fewer than 50% motile spermatozoa with adherent particles.

Sperm morphology is also considered part of the standard semen analysis. The WHO (1999), however, is not as clear as to the percentage of sperm forms necessary to produce a normal result. Because the WHO is currently revising their standards concerning sperm morphology, they do not give a specific number but note that "as sperm morphology falls below 15% normal forms using the methods and definitions described in this manual, the fertilization rate of in vitro decreases."

Sperm morphology may help provide the clinician with added information for advising couples facing

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infertility. For this tool to be reliable, however, it must be reproducible and easily interpreted. Unfortunately, sperm morphology is a subjective analysis; therefore, it is prone to technician bias. As a result, one laboratory may deem a male to be fertile based on one set of morphologic criteria whereas another laboratory may consider this individual infertile based on a different set of morphology standards (Neuwinger *et al.*, 1990).

When developing a technique for evaluating sperm morphology, potential error should be considered. There are at least three areas in which error can occur: staining of spermatozoa, use of Computer-Assisted Semen Analysis (CASA), and variability of technicians.

Staining Technique

There are many stains that a laboratory can use to facilitate the study of sperm. Some of these stains include Diff-Quik fixative (Kruger *et al.*, 1988), Papanicolau (Kruger *et al.,* 1986; WHO, 1999), Shorr (WHO, 1999), Wright Giemsa (Menkveld *et al.*, 1990) and Spermac® (Chan *et al.*, 1996). Unfortunately, problems have been identified with the use of these various stains. Diff-Quik may have background staining and increase the size of the sperm head (WHO, 1999). With the Wright Giemsa stain, sperm heads without acrosomes may appear normal (Menkveld *et al.*, 1990). The World Health Organization (1999) recommends Papanicolau stain, which is used the most widely in laboratories. It allows staining of the acrosomal region and post-acrosomal region, cytoplasmic droplets, mid-piece and the tail (WHO, 1999); however, it is very time consuming to perform. In the end, the method for use of staining should be one that is most beneficial for the lab (Davis *et al.*, 1995).

Computer-Assisted Semen Analyzers

Computer-Assisted Semen Analyzers can be used to help analyze sperm morphology. Such instruments include Cell Form Human (Motion Analysis Corporation, Santa Rosa, CA), Cell Soft Morphologizer II (Cryo Resources LTD., New York, NY), Leitz-TAS-Plus (E. Leitz, Rockleigh, NY), Hamilton Thorne IVOS (Hamilton Thorne Research Inc., Beverly, MA) and FERTECH Morphologizer (FERTECH, Norfolk, VA). Computer-assisted semen analyzers locate the fields of sperm, recognize sperm heads, and perform measurements. While most computers are automatic, they can be operated manually.

The CASA is not without problems. For example, with the CASA, a technician must use correct illumination and focus in a vibration-free workplace for the computer to consistently read sperm morphology (Coetzee *et al.*, 1999). Furthermore, staining variation between cells can cause inaccurate recognition or measurements and head digitization errors (Davis and Gravance, 1993). In addition, the sperm density and uniformity on the slide affects the computers ability to identify sperm in a timely manner (Davis and Gravance, 1993). Also, different stains can cause head digitization error. Finally, some CASA systems are very slow and may require more than 3 h to read 200 sperm (Garrett and Baker, 1995).

Technician Variation

Internal quality control would be helpful in maintaining accurate morphology results. Clements *et al.* (1995) implemented a process in which the quality control was integrated into the normal workflow so as not to be disruptive. This continuous quality control and proper training of technicians can result in repeatable sperm morphology data. Studies by Cooper *et al.* (1999) and Franken *et al.* (2000) demonstrated that a high level of accuracy could be obtained for morphology assessment between laboratories if an intensive training program with quarterly quality controls can be maintained.

An assessment of means can be useful if the patient population does not vary greatly. This assessment can provide a potential warning if the morphology percentage declines, but the other parameters do not. Such a decline can indicate a change in the analysis of sperm morphology by lab personnel, but not necessarily a decrease in normal sperm morphology.

Summarized within this text are the results of three research projects focusing on mammalian sperm morphology. The first focus is to examine the effect of staining protocol on sperm morphology as measured by CASA analysis (Hamilton-Thorne Integrated Visual Optical System [IVOS]; Version 10.0; Hamilton-Thorne Research, Beverly, MA). The second focus is an attempt to establish a set of sperm morphology values for use as a laboratory reference for CASA. The last focus is to determine the repeatability between novice and seasoned technicians for sperm morphology with a secondary aim to determine the normal range for sperm morphology among men in the Greenville, South Carolina area.

MATERIALS AND METHODS

Staining Technique

Slides created from 15 beef bull ejaculates were chosen at random and stained in three different protocols. Morphology slides were prepared as described from the Hamilton Thorne manual (Hamilton Thorne, Beverly, MA). Briefly, the fresh bovine semen specimens were washed in 1% BSA:PBS solution and approximately 3 million spermatozoa were smeared on a glass slide. Slides were allowed to dry on a 37°C slide warmer prior to staining with one of the three regimes listed in Table I. After staining, slides were cover slipped, and analyzed with the use of the Metrix version 2.7 software (Hamilton Thorne, Beverly, MA).

CASA

Members of the Andrology Laboratory at the Greenville Hospital System examined 42 human morphology slides to determine a laboratory standard for normal sperm criteria. Fixed sperm were examined with the use of the IVOS Metrix program. After the laboratory staff had selected and captured 400 normal sperm images, values associated with these sperm were calculated (means and standard deviations).

A second test was performed using proficiency testing slides from the American Association of Bioanalysts (AAB; St. Louis, MO). We compared our newly obtained IVOS ranges for normal sperm morphology to a technician's evaluation of normal morphology using a standard compound microscope. For computer analysis, slides were searched for sperm and evaluated with the IVOS Metrix program. Once a sperm was observed, the program evaluated and labeled the cell on the screen. Each slide was searched until 200 sperm were labeled. Furthermore, a technician reviewed each sperm the program labeled and sperm not correctly labeled were removed before analysis. Slides were evaluated in duplicate.

Table I. Staining of Bovine Spermatozoa Using Three Different Methods

Group	Diff-quik	Time (min)	Temperature (°C)
1	Fixative	0.5	23
	Stain	1	23
	Counter stain	1	40
2	Fixative	0.5	23
	Stain	2	40
	Counter stain	2	40
3	Fixative	0.5	23
	Stain	5	40
	Counter stain	5	40

The two IVOS replications were averaged for percent normal sperm and compared to a technician's determination of normal sperm morphology for the same slide. In addition, both were compared to the AAB proficiency testing results. Computer evaluation of a slide required 30–60 min per replication, whereas technician evaluation required 3–5 min per slide.

Coefficients of variation (CV) were calculated for sperm morphology readings among computer-generated results (Comp), our own morphology results (REI), and those released from the American Association of Bioanalysis (AAB). The analyses were divided into the following groups: CV between counts within the computer-generated analysis, CV among the three types of morphology readings, and CV between REI and AAB morphology readings.

Technician Variation

Twenty semen donors were chosen from a group of men responding to a clinical study advertisement or recruited by word of mouth. The participants were required to have fathered a child in the last 2 years and to be between 20 and 45 years of age. Prior to semen collection, the participants were required to abstain from ejaculation for at least 48 h. Semen was collected via masturbation at our clinic and processed within 1 h. The semen analysis was performed manually or with the aid of a CASA.

Slides for sperm morphology evaluation were prepared from an aliquot of the raw semen washed in PBS and centrifuged with the resulting pellet providing approximately 3 million sperm per slide. The slides were air-dried and stained with Wright stain. Once the stain had dried, a cover slip was placed on to the slide with Permount®.

To determine variation among and within laboratorians, five technicians (two experienced and three novice) read 20 slides in duplicate. The slides were re-randomized and re-evaluated a week later. This was repeated to have a total of six evaluations per slide. The means and 95% confidence intervals for the normal sperm percentage were determined for each technician.

Table II. Size of Bovine Spermatozoa When Exposed to Three Different Methods of Staining

Group #	Type	Major axis (μm)	*SD*	Minor axis (μm)	*SD*	Area (μm^2)	*SD*	Elong (%)	*SD*	Perimeter (μm)	*SD*
1	Normal	8.87	0.23	4.48	0.16	29.37	1.73	50.66	1.76	22.15	0.55
$n = 208$	Abnorml	7.81	0.49	4.12	0.37	22.85	3.86	52.90	5.15	20.42	0.95
	Mean	8.34[a]	0.36	4.30[a]	0.26	26.11[a]	2.79	51.78[a]	3.45	21.28[a]	0.75
2	Normal	9.12	0.46	4.88	0.26	35.02	2.76	53.57	3.06	23.26	0.91
$n = 505$	Abnorml	9.39	0.71	5.16	0.40	37.68	4.39	55.16	4.43	24.03	1.47
	Mean	9.26[b]	0.59	5.02[b]	0.33	36.35[b]	3.58	54.37[b]	3.74	23.65[b]	1.19
3	Normal	9.47	0.41	4.94	0.23	36.86	2.41	52.26	2.81	23.93	0.79
$n = 465$	Abnorml	9.75	0.58	5.23	0.32	39.70	3.36	53.89	4.06	24.76	1.09
	Mean	9.61[c]	0.49	5.08[c]	0.27	38.28[c]	2.89	53.08[c]	3.43	24.34[c]	0.94

[abc]Groups with different letters within the same column are significantly different ($P < 0.05$).

RESULTS

Staining Technique

A brief summary of the bull data is presented in Table II. When the extended stain and counter-stain time are coupled with additional heat, there is a tendency to improve ability to read spermatozoal morphology when compared to Hamilton-Thorne's recommended protocol (Group 1). However, the longer the sperm remained in the stain and counter stain, the larger the major and minor axis became. These increases in axes meant a total area increase for those spermatozoa that remained in the stain and counter stain longer.

CASA

Table III represents IVOS Metrix program factory ranges for normal human sperm and the ranges calculated from our own laboratory observations.

Table IV denotes the second test that describes percent normal human sperm morphology and coefficients of variation of the 12 AAB slides using the IVOS. For CV between or among replications, the standard accepted range for our laboratory is to be no greater than 15%. The standard accepted range between trials occurred 8 out of 12 times (67%) for the 12 counts; otherwise the CV was above 15%.

As indicated in Table V the CV was outside this range for the 12 slides that were observed by the computer, our laboratory (REI), and as reported by the AAB.

Table VI compares the CV for our laboratory (REI) and as reported by the AAB. The standard accepted range of $\leq$15% for the CV only occurred 3 out of 12 times (25%).

Table III. Parameter Settings for the IVOS as Determined by the Manufacturer (Hamilton Thorne) and by Reproductive Endocrinology and Infertility (REI)

Parameter	Hamilton-Thorne range		REI range	
Major axis (μm)	4.5	5.5	4.0	5.0
Minor axis (μm)	2.5	3.5	2.5	3.5
Area (μm)	8.8	15.0	8.0	30.0
Elongation (%)	45.0	78.0	60.0	80.0
Perimeter (μm)	10.0	14.0	8.0	13.0
Acrosome (%)	40.0	70.0	35.0	55.0

Technician Variation

The average normal sperm morphology for the two seasoned evaluators was 10.4% and 10.8%. The average normal sperm morphology for the three novice evaluators was 7.3%, 7.9% and 15.0% (Fig. 1). The two seasoned evaluators reported the percent normal range for sperm morphology among the men as

Table IV. Percent Normal Human Sperm Morphology and Coefficients of Variation (CV) for Sperm Morphology Readings Between Counts Within the Computer Generated Analysis

Proven donors	Replication A (%)	Replication B (%)	*SD*	*CV*
1	33.8	31.8	1.4	4.4
2	28.0	16.0	8.5	53.0
3	27.9	29.1	0.8	2.9
4	27.5	24.3	2.3	9.3
5	26.9	26.8	0.1	0.3
6	23.6	28.8	3.7	12.8
7	21.8	29.8	5.7	19.0
8	20.4	27.8	5.2	18.8
9	19.8	18.8	0.7	3.8
10	19.1	19.4	0.2	1.1
11	10.2	17.5	5.2	29.5
12	3.9	4.4	0.4	8.0

Table V. Percent Normal Human Sperm Morphology and Coefficients of Variation (CV) Among Three Types of Human Sperm Morphology Readings

IVOS[a]	REI[b]	AAB[c]	Mean	*SD*	*CV*
32.8	14	6.4	17.7	13.6	76.6
28.5	18	13.4	20.0	7.7	38.8
26.8	35	14.1	25.3	10.5	41.6
26.2	19	15.5	20.2	5.5	27.0
25.9	13	14.0	17.6	7.2	40.7
25.8	4	7.0	12.3	11.8	96.3
24.1	25	9.2	19.4	8.9	45.7
22.0	18	15.3	18.4	3.4	18.3
19.3	22	8.4	16.6	7.2	43.5
19.2	6	1.7	9.0	9.1	101.7
13.8	10	2.9	8.9	5.5	62.2
4.2	17	8.8	10.0	6.5	64.8

[a]IVOS: Interoptical Visual Optics System, Hamilton Thorne, Beverly MA.
[b]REI: Five technicians from Reproductive Endocrinology and Infertility at the Greenville Hospital System in Greenville, South Carolina.
[c]AAB: American Association of Bioanalysts, St Louis, Missouri.

9.3% to 11.9%. The differences noted between the seasoned and novice technicians were not statistically significant.

DISCUSSION

This study shows the potential variation in determining sperm morphology that exists in staining techniques as well as in computer- and technician-generated results. We have shown that even with the use of the same stain, altering exposure time to the stain and the counter stain will significantly change sperm morphology. In our laboratory, computer generated results for sperm morphology have a large coefficient of variation. Furthermore, individual technicians, especially novices, can contribute to the amount of variation in assessment of sperm morphology.

Table VI. Coefficients of Variation (CV) for Percent Normal Human Sperm Morphology Readings Between Reproductive Endocrinology and Infertility (REI) and American Association of Bioanalysts (AAB)

REI	AAB	Mean	*SD*	CV
6	1.7	3.9	3.0	79.0
10	2.9	6.5	5.0	77.8
14	6.4	10.2	5.4	52.7
4	7.0	5.5	2.1	38.6
22	8.4	15.2	9.6	63.3
17	8.8	12.9	5.8	44.9
25	9.2	17.1	11.2	65.3
18	13.4	15.7	3.3	20.7
13	14.0	13.5	0.7	5.2
35	14.1	24.6	14.8	60.2
18	15.3	16.7	1.9	11.5
19	15.5	17.3	2.5	14.3

It should be noted that different staining protocols produce different mean head sizes; therefore, if CASA is to be used, the parameters for evaluating spermatozoa must be set according to the stain the laboratory uses. Even if a manual method is used to determine sperm morphology, the reader must be cognizant of the stain being used and the potential for changes in the size of the sperm heads.

Two problems noted with the IVOS Metrix program for sperm morphology were the amount of time needed to perform the analysis and the accuracy of the results. In some cases, the computer analysis of one slide for morphology encompassed an hour, whereas a technician can manually evaluate a morphology slide in less than 5 min. As for the accuracy of the analyses, the computer incorrectly evaluated 42.4% (2585/6093) of the sperm observed.

Although a routine semen analysis was performed for the 20 participants, the only variable evaluated in this study was morphology. The other variables in the semen analysis fell within the normal range according to WHO (1999) standards. In this study, the normal sperm morphology range for the men reported by the seasoned analyzers was 9.3 and 11.9. This range is below the reference of normal men ($\geq$15%) described by Kruger and coworkers (1986) and the WHO (1999).

Freund (1966) and Eliasson (1971) made some of the earliest contributions towards standardization of sperm morphology. Further advancements on this idea were reported by Kruger who used "strict" sperm morphology to demonstrate that 0 to 14% normal forms would produce fertilization, but did not yield a pregnancy when intracytoplasmic sperm injection (ICSI) was performed (1986). However, in a later report, Kruger has noted that if ICSI is performed, fertilization as well as pregnancy can occur with as little as 0 to 4% normal forms (2004). Thus, while this low percentage of normal forms can be considered "abnormal," the male can still be considered fertile according to Jeyendran (2003).

In our laboratory, mechanisms will have to be developed to improve the repeatability of these procedures if the size of the CV is to be reduced, especially for the novice reader of sperm morphology. Training should be a continuous process in which all analyzers evaluate and discuss results. This openness will lead to repeatable and interpretable results by all technicians and provide the most informative results to the referring physicians and their patients.

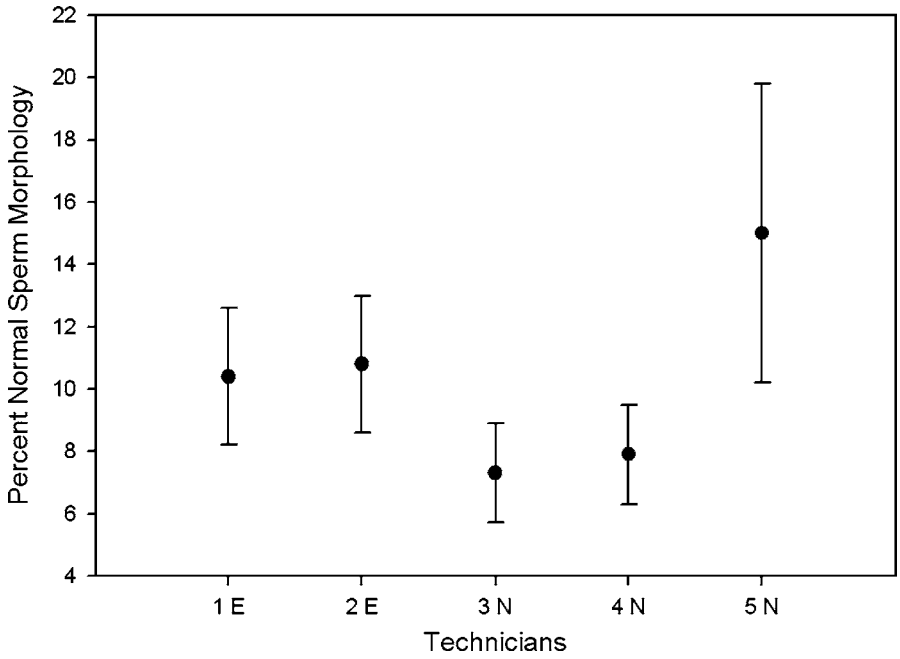

Fig. 1. Mean normal sperm morphology percentage and 95% confidence interval for five technicians [2 experienced (E) and 3 novice (N)] evaluating 20 specimens repeated 6 times over a 6-week period of time.

In conclusion, until all laboratories adhere to the universal standard set by WHO to evaluate sperm morphology, laboratories will have to rely on their own quality control measure to provide repeatable results. From these data, it would appear that stain techniques and technician variation can, and do, alter the results. While the use of CASA to aid in objective semen evaluation is theoretically a good idea, our data indicate that computer evaluation of sperm morphology is time consuming and lacks accuracy. Finally, from our study of conducting sperm morphology on men who have recently fathered a child, normal sperm morphology appears to be closer to 10% as opposed to 15% as described by Kruger *et al.* (1986).

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