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Longitudinal Study of Semen Quality of Unexposed Workers: SPERM HEAD MORPHOMETRY

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As part of a longitudinal study of human semen characteristics of unexposed workers, sperm head measurements were made using image analysis (Image Technologies Corp., Deer Park, NY). Morphometry was conducted on monthly samples from 45 men for 9 months. Measurements of area, perimeter, length, width, the width-length ratio, and the oval factor (4 [pi] area/ perimeter^2) of 100 sperm heads per sample were obtained. The variability within a sample, between monthly samples from the same individual, and between individuals were calculated for each parameter. Tolerance intervals for each parameter were calculated, and are expected to contain 75% of all individual measurements. Similar intervals were calculated for the means and coefficients of variation of a semen sample. The largest source of variability was attributed to individual cell variation within a semen sample.

Key words: morphology, morphometry; sperm head, statistics, tolerance intervals.

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Sperm morphology has been a traditional semen characteristic for assessing the fertility status of men. During the past 30 years, several morphology schemes have been presented for the assessment of normal and abnormal sperm morphologies. While some sperm obviously fit into specific classifications, there are many sperm of transitional shapes and sizes that make repeatability of classification difficult for a given technician and even worse between laboratories (MacLeod, 1951; Freund, 1966; Fredricson, 1979; Hanke, 1981). With the recent advances of computerized image analysis, several methods of sperm morphometry have been introduced (Schmassmann et al, 1979; Katz et al, 1981; Schrader et al, 1984; Jagoe et al, 1986; DeStefano et al, 1987; Turner et al, 1988; Moruzzi et al, 1988). These morphometric analysis systems provide objective assessments of individual sperm head size and shape; however, comparisons between measurements from different analysis systems should be avoided (Turner et al, 1988).

Sperm morphometry is now routinely used as part of the assessment of reproductive hazards to male workers (Schrader et al, 1987). A recent study by Ratcliffe et al (1987) reported a significant reduction in sperm head width with ethylene dibromide exposure of male workers. Therefore, information on the population stability and variability of these parameters is needed. This report evaluates the variation of sperm size and shape within an ejaculate, between ejaculates from the same man, and between healthy young men without known toxicant exposure.

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Methods

Sperm morphometry was conducted as part of a longitudinal study of human semen characteristics. The basic study design has been presented elsewhere (Schrader et al, 1988). Briefly, monthly semen samples were collected by masturbation from 45 men (ages 25–35 years) over a 9 month period. The men were from various occupations (both blue collar and white collar) in the Cincinnati area, and all were employed (or were graduate students) at the onset of study. Eighty percent of the men consumed alcohol, 52% smoked (pipe, cigar, or cigarettes), and 46% had fathered at least one pregnancy. The population is described in more detail in Schrader et al, 1988. Medical and occupational histories were collected from each participant, and none reported having a fertility problem or exposure to known reproductive toxicants.

Morphometric Analysis

When the semen samples were delivered to the laboratory, four air dried microscope slides were prepared, fixed in ethanol, and stained with Papanicolaou stain (Belsey, 1980). Morphometry measurements were made with an automatic image analysis system (Model 3000 Image Analyzer, Image Technology Corp., Deer Park, NY) interfaced with a Zeiss microscope equipped with a 63x objective and a 2x enlargement lens. The image analysis system was calibrated using a stage micrometer. The system allowed the user to edit the image to ensure that only the sperm head was being analyzed. One slide from each ejaculate was analyzed by assessing 100 arbitrarily selected sperm heads. The computer calculated the length, width, area, and perimeter of each sperm head. Further calculations for determining sperm head size were the ratio (width/length) and the oval factor (4 pi [area]/ perimeter^2).

Statistical Analysis

For each of the six morphometric parameters, data from individual cells were analyzed using PROC NESTED and PROC ANOVA in Statistical Analysis Systems (SAS Institute Inc., 1985). Individual men were treated as a main effect. Individual semen samples were a second factor nested within the first. Both factors were treated as random effects. From this analysis, three components of variance were estimated: between men, between semen samples within men, and between sperm cells within semen samples.

Means and coefficients of variation of individual semen samples were computed for each morphometry variable and analyzed to estimate the components of variance using PROC NESTED and PROC ANOVA. The individual man was the main effect factor and was treated as a random effect for this analysis. Two sources of variation were estimated: between men and between semen samples.

For all the analyses (individual sperm cells, ejaculate means, and ejaculate coefficients of variation) the assumptions of normality and equal variances were checked using the appropriate residual plots. Only ejaculate coefficients of variation required a logarithm transformation to satisfy the needed assumptions.

For each morphometric parameter, total standard deviation, total sampling error, and tolerance intervals were estimated from the components of variance obtained from each analysis (eg, individual cells, semen sample means, and semen sample coefficients of variations). Total

TABLE 1. Components of Variation of Individual Sperm Head Measurements

	Length		Width	
	Component	Percent of Total	Component	Percent of Total
V_{men}	0.068	13	0.009	5
$V_{samples}$	0.048	9	0.021	13
V_{cells}	0.423	78	0.137	82
Total	0.539	100	0.167	100

	Area		Perimeter	
	Component	Percent of Total	Component	Percent of Total
V_{men}	0.31	7	0.39	10
$V_{samples}$	0.70	17	0.33	8
V_{cells}	3.16	76	3.34	82
Total	4.17	100	4.05	100

	Width/Length Ratio		Oval Factor	
	Component	Percent of Total	Component	Percent of Total
V_{men}	0.0015	12	0.0007	6
$V_{samples}$	0.0003	2	0.0010	8
V_{cells}	0.0107	86	0.0103	86
Total	0.0125	100	0.0120	100

TABLE 2. Tolerance Intervals for Sperm Head Morphometry Individual Sperm Head Measurements

	Grand Mean	Total Sampling Error	Total Standard Deviation	Lower Limit	Upper Limit
Length (μm)	4.53	0.041	0.756	3.6	5.5
Width (μm)	2.85	0.016	0.416	2.3	3.4
Area (μm²)	8.72	0.096	2.089	6.1	11.3
Perimeter (μm)	12.69	0.10	2.06	10.1	15.3
Width/length	0.641	0.006	0.120	0.50	0.79
Oval factor	0.688	0.004	0.112	0.55	0.84

These tolerance intervals have 95% confidence of containing 75% of the individual cell measurements in the population.

standard deviation was an estimate of the standard deviation: 1) of a measurement on an individual cell across all samples and all men, 2) of a single semen sample mean across all men, and 3) of a single semen sample coefficient of variation across all men. Total sampling error was an estimated standard deviation measuring the combined sampling error associated with the sampling scheme of 45 men with nine samples per man. The tolerance interval combined total standard deviation and total sampling error to produce an interval that contained a desired portion of the population. For this study, a tolerance interval was calculated which we are 95% confident will contain 75% of the population. In other words, this is an interval which we are 95% confident will contain the 12.5 percentile of the population through the 87.5 percentile. Formulas for each of these quantities are shown in the appendix and a more complete explanation is reported by Simon et al (unpublished).

Results

The components of variance for the morphometry measurements of individual sperm cells are presented in Table 1. For all measurements, variation between cells dominated, accounting for 76–86% of the total variation. This indicates that a wide range of shapes can be found in any semen sample from any man. Surprisingly, variation from one man to another was small, accounting for only 5–13% of the total variation.

Table 2 presents tolerance intervals for individual sperm cells. These are intervals that we are 95% confident will contain 75% of all individual sperm cell measurements across all samples and all men. For example, the length of most sperm cells can be expected to lie between 3.6 μm and 5.5 μm.

The components of variance for the morphometry measurements of sperm head means are presented in Table 3. Table 4 presents tolerance intervals of average values for semen samples determined from measurements on 100 sperm heads. These are intervals that we are 95% confident will contain 75% of all such averages across all men. For example, the average sperm head length for most semen samples can be expected to lie between 4.0 μm and 5.1 μm. Each of the intervals in this table is narrower

TABLE 3. Components of Variation of Sperm Head Means

	Length		Width	
	Component	Percent of Total	Component	Percent of Total
V_{men}	0.068	56	0.0090	28
$V_{samples}$	0.052	44	0.0227	72
Total	0.120	100	0.0317	100
	Area		**Perimeter**	
	Component	Percent of Total	Component	Percent of Total
V_{men}	0.31	30	0.387	52
$V_{samples}$	0.73	70	0.359	48
Total	1.04	100	0.746	100
	Width/Length Ratio		**Oval Factor**	
	Component	Percent of Total	Component	Percent of Total
V_{men}	0.00148	77	0.00075	41
$V_{samples}$	0.00043	23	0.00106	59
Total	0.00191	100	0.00181	100

TABLE 4. Tolerance Intervals for Sperm Head Morphometry Semen Sample Means

	Grand Mean	Total Sampling Error	Total Standard Deviation	Lower Limit	Upper Limit
Length (μm)	4.53	0.040	0.399	4.0	5.1
Width (μm)	2.85	0.016	0.195	2.6	3.1
Area (μm²)	8.72	0.093	1.123	7.2	10.2
Perimeter (μm)	12.69	0.096	0.98	11.3	14.0
Width/length	0.641	0.006	0.053	0.57	0.71
Oval factor	0.688	0.004	0.048	0.62	0.75

These tolerance intervals have 95% confidence of containing 75% of the semen sample mean measurements in the population.

than the corresponding interval in Table 2. Averaging 100 sperm measurements removes much of the variation between sperm cells. One should be cautious when using these intervals. These intervals are for averages of 100 sperm heads and need minor adjustments if one averages a different number of sperm cell measurements. Tolerance intervals for an average of 25 cells, for example, would be slightly wider than intervals reported for an average of 100 cells (see appendix).

For morphometry measurements, Katz et al (1986) reported that the per-ejaculate variability rather than central tendency maximized the differences between fertile and infertile men. Therefore, we calculated components of variance and tolerance intervals for the coefficient of variation (CV) of a semen sample of 100 sperm heads (Tables 5 and 6). These are intervals that we are 95% confident will contain 75% of all coefficients of variation across all men. The CV area has a tolerance interval ranging up to 25.8%, much higher than the other morphometry variables. Thus, a CV of 22% would be uncommon for length or width, but not for area. With the exception of area, the lower limits of these tolerance intervals range from 10–13% and the upper limits range from 17–20%.

Discussion

Sperm head morphometry was determined for monthly ejaculates from 45 unexposed men. Tolerance intervals were calculated for both individual cells and sample means for each morphometry variable. Tolerance intervals were calculated rather than confidence intervals because we are interested in classification of individual semen samples or individual sperm cells as typical of or atypical of a large portion of the population. A confidence interval places limits on the average sperm cell area of an average semen sample from an average man. Few men actually are average. Men

TABLE 5. Components of Variation of Sperm Head Coefficients of Variation

	Length		Width	
	Component	Percent of Total	Component	Percent of Total
V_{men}	0.0030	16	0.0131	43
$V_{samples}$	0.0157	84	0.0176	57
Total	0.0187	100	0.0307	100
	Area		**Perimeter**	
	Component	Percent of Total	Component	Percent of Total
V_{men}	0.0119	34	0.0016	5
$V_{samples}$	0.0235	66	0.0288	95
Total	0.0354	100	0.0304	100
	Width/Length Ratio		**Oval Factor**	
	Component	Percent of Total	Component	Percent of Total
V_{men}	0.0112	53	0.0091	20
$V_{samples}$	0.0099	47	0.0373	80
Total	0.0211	100	0.0464	100

TABLE 6. Tolerance Intervals for Sperm Head Morphometry Semen Sample Coefficient of Variation

	Grand Mean	Total Sampling Error	Total Standard Deviation	Lower Limit Percent	Upper Limit Percent
Length	14.0	1.01	1.16	11.5	17.0
Width	12.6	1.02	1.22	9.6	16.4
Area	19.5	1.02	1.23	14.8	25.8
Perimeter	13.9	1.01	1.21	10.9	17.6
Width/length	16.0	1.02	1.18	12.7	10.6
Oval factor	14.4	1.02	1.26	10.6	19.6

Note: Values in this table are back transformed from a log scale to the original scale of measurement. These tolerance intervals have 95% confidence of containing 75% of the semen sample coefficients of variation of measurements in the population.

tend to be either above or below average due to a variety of demographic and physiologic characteristics. Semen samples or individual sperm cells are rarely average but vary around the mean. A confidence interval would ignore variation from man to man, semen sample to semen sample, and sperm cell to sperm cell (the net effect is referred to as total standard deviation), and assesses the impact of total sampling error only. Tolerance intervals account for both total standard deviation and total sampling error. Tolerance intervals, therefore, provide information which can help to characterize an individual man's results, rather than a prediction of the values for a nonexistent average man.

Tolerance intervals for individual sperm heads could possibly form the basis for an objective and reproducible morphology classification scheme. An individual cell would be labeled as typical if its morphometry measurements lie inside certain tolerance intervals, and atypical otherwise. These atypical cells could be further classified if necessary. A sperm cell with a width less than 2.3 μm could be labeled atypically narrow and a cell with an area greater than 11.3 μm^2 could be labeled atypically large area. Other studies on populations from other geographic locations (and climates) are needed before proposing such a classification.

In Table 1, the large relative size of between cell variation was noted. The impact of this source of variation can be readily minimized by the statistical planning of future occupational field studies as the following examples will illustrate.

Suppose we want to estimate the average sperm cell area in a specific subpopulation of workers. We recruit a certain number of men (n_{men}) and obtain from each a certain number of semen samples ($n_{samples}$). In each sample, we measure a certain number of sperm cells (n_{cells}). The average area would have a standard error equal to:

$$V_{men}/n_{men} + V_{samples}/(n_{men} * n_{samples}) + V_{cells}/(n_{men} * n_{samples} * n_{cells})$$

where $V_{men} = 0.309$, $V_{samples} = 0.702$, and $V_{cells} = 3.156$, which are the components of variance between men, samples, and cells as reported in Table 1.

A typical study might have $n_{men} = 40$, $n_{samples} = 2$, and $n_{cells} = 100$. In this case the standard error would be 0.13. By quadrupling the number of men, the standard error would be cut in half. By quadrupling the number of samples, the standard error would be cut by 15%. By quadrupling the number of cells, the standard error would only be lowered by 1%. As long as the number of cells counted (per sample) is 50 or more, the relative contribution of variation between cells is negligible.

The tolerance intervals presented here provide information on the range of cells found in ejaculates from unexposed male workers. These intervals for individual cells, for semen sample means, and for semen coefficients of variation may prove valuable in labeling results from an individual man as typical or atypical. Tolerance intervals for individual cells may also prove valuable in forming an objective classification of sperm head size and shape.

Evaluating the components of variation provides useful information about the morphometry parameters. In the evaluation of the components of variation of individuals cells (Table 1), at least 76% of the variation for a morphometry parameter was observed as cell to cell variation. This suggests that each ejaculate contains many sizes and shapes of sperm heads. In the evaluation of the components of variation of sperm head means (Table 3), most parameters had roughly equivalent man to man and sample to sample variation. In Table 3, the (V_{men}) is equal to the interclass correlation; therefore, a large man to man variability (V_{men}) is indicative of one sample being representative of that individual;

whereas, a low (V_{men}) value is indicative of a greater need for repeat sampling.

Although it is difficult to quantify seasonal variation in a nine-month study, indications are that seasonal variations are small. If there is a seasonal component, $V_{samples}$ from the longitudinal study might be too large, making the tolerance intervals slightly too wide. Further work, however, is needed to more fully assess the influence of geographic and seasonal variation on sperm head morphometry.

From a biological viewpoint, each morphometric parameter may provide important information about different aspects of spermatogenesis. It is not, therefore, the intent of this report to suggest that one parameter is better than another, but to provide researchers with information on the variability of each parameter in order to design the most powerful study for assessing insults to the male reproductive system.

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The 1990 Annual Meeting of the American Society of Andrology
Student Affairs Committee Announcement

Hotel Arrangements: The student room rate at the Marriott Downtown Hotel will be $72.00/room/night for 1–3 people/room. A clearing house has been organized to facilitate room sharing. Contact Dr. Susan Rothmann, 216-444-2488 for assistance.

Student Soiree: 5:00–6:00 p.m. Friday evening, April 6, Guest of Honor, Tracy Rankin, 1989 New Investigator Award winner.

Placement Service Board: Positions available and positions wanted will be posted. To register in advance, contact William Baird, PhD, 614-442-0122.

Student Colloquium: 8:30–10:00 p.m. Saturday evening, April 7, Speaker: Philip Troer, MD, President, International Society of Andrology. Topic: What in the world is going on?

Appendix—Formulas for Tolerance Intervals

In this appendix, we will briefly outline tolerance intervals and provide some examples of their computation. The formulas presented here are an adaptation of an idea in Wald and Wolfowitz (1946).

For the case of individual cells, we have three components of variation, V_{men}, $V_{samples}$, and V_{cells} cells. The total standard deviation can be computed as:

$$TSD = (V_{men} + V_{samples} + V_{cells})^{0.5}$$

but the values for total standard deviation in the tables are actually an upper bound for total standard deviation. This upper bound is based on a chi-squared distribution with degrees of freedom estimated by Satterthwaite's approximation (Montgomery, 1984). Total sampling error for the individual cell's confidence interval is computed as:

$$TSE = [V_{men} / n_{men} + V_{samples} / (n_{men} * n_{samples}) + V_{cells} / (n_{men} * n_{samples} * n_{cells})]^{0.5}$$

for the case where the same number of cells were counted for each sample and the same number of samples were provided by each man. For the case where the same number of cells counted per sample and the number of samples provided by each man differ, as the case was here, change the denominator of the second term to the total number of samples analyzed for all men (399 in this case), and the denominator of the third term to the total number of cells counted for all samples and men (39,900 in this case). This provides a reasonable approximation for TSE. The exact formula is too complex to be shown here.

The tolerance interval specifies a proportion of the population. It contains 1 minus gamma (in this report 75%) and a level of confidence 1 minus alpha (in this report 95%). To compute the tolerance interval we use the formula:

$$GM \pm [(Z_{gamma/2} * TSD) + (Z_{alpha/4} * TSE)]$$

where TSD is the total standard deviation, TSE is the total sampling error, and Z represents an upper percentile from the normal distribution. Note that alpha/4, rather than alpha/2, is used in this formula. This reflects the use of the Bonferroni inequality when bounding sampling errors for the mean as well as the total standard deviation. For example, a 95% tolerance interval for 75% of sperm lengths is computed as:

$$4.53 \pm [(Z_{0.125} * 0.756) + (Z_{0.0125} * 0.041)]$$

where $Z_{0.125} = 1.1503$ and $Z_{0.0125} = 2.2416$. To compute a tolerance interval for 90% of the population (instead of 75%), replace $Z_{0.25}$ with $Z_{0.05}$, which would widen the interval from 3.2 to 5.9. Changing of the confidence level (eg, 95–99%) involves more computations and need not be discussed here.

The calculation of the tolerance interval for a semen sample average proceeds similarly. There are only two components of variation, V_{men} and $V_{samples}$. The $V_{samples}$ for the mean is slightly larger than $V_{samples}$ for the individual cells because we add to it, $V_{cells}/100$, the total sampling error for an average of 100 cells. The other source of variation, V_{men}, is the same as for the individual cells.

Total standard deviation for a semen sample mean is:

$$TSD = (V_{men} + V_{samples})^{0.5}$$

and the upper bound for the quantity is computed as in the previous section. Total sampling error is:

$$TSE = (V_{men} / n_{men} + V_{samples} / (n_{men} * n_{samples})^{0.5}$$

for the case of equal numbers of samples per man. For the case of unequal numbers, replace the denominator of the second term by the total number of samples for all men (399 in this case) to get a reasonable approximation for TSE. The formula for tolerance intervals remains the same as before.

For example, a 95% tolerance interval for 75% of the mean lengths is:

$$4.53 \pm [(Z_{0.125} * 0.399) + (Z_{0.0125} * 0.040)]$$

or 4.0 μm to 5.1 μm. Modification to percentages other than 75% involve changing $Z_{0.125}$ to the appropriate normal percentile.

Estimating a tolerance interval for a mean of a different number of cells would require some adjustments. For example, $V_{samples}$ for an average of 25 cells would be computed by adding $V_{cells}/25$ to $V_{samples}$ from individual cells (Table 1).

Tolerance intervals for coefficient of variation are similar to the above except that they need to be transformed back to the original scale of measurement.

Total standard deviation for a semen sample CV is:

$$TSD = \exp [(V_{men} + V_{samples})^{0.5}]$$

and the upper bound for this quantity is computed as above. Total sampling error is:

$$TSE = \exp [(V_{men} / n_{men} + V_{samples} / (n_{men} * n_{samples})^{0.5}]$$

for the case of equal numbers of samples per man. For the case of unequal numbers, replace the denominator of the second term by the total number of samples for all men to get a reasonable approximation for TSE. The formula for the tolerance interval becomes:

$$GM/(TSD^{**}Z_{1-gamma/2} * TSE^{**}Z_{1-alpha/4})$$

$$GM*(TSD^{**}Z_{1-gamma/2} * TSE^{**}Z_{1-alpha/4})$$

where ** denotes raising to a power

The following is an example of computing a tolerance interval for a coefficient of variation. Suppose, for example, that we wish to compute a 95% tolerance interval for 75% of the length coefficients of variation. The limits of this interval would be:

$$14.0/(1.16^{**}Z_{0.125} * 1.01^{**}Z_{0.0125})$$

$$14.0*(1.16^{**}Z_{0.125} * 1.01^{**}Z_{0.0125})$$

which yield the interval 11.5–17.0%. To change from 75% of the population to any other proportion, simply change $Z_{0.125}$.

15th Annual Meeting
American Society of Andrology
and
Postgraduate Course
April 6–9, 1990
Downtown Marriott Hotel
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The scientific program will feature lectures on pediatric andrology, techniques in andrology research, the role of growth factors and oncogenes in reproduction, *in vitro* fertilization, and embryo transfer. Platform and poster presentations of new research are also scheduled.

The theme for the Postgraduate Course, to be held Friday, April 6, will be "Immunologic Aspects of Infertility."

For information contact Tu Lin, MD, Program Chairman (803/776-4000), or Howard Nankin, MD, Local Arrangements Chairman (803/733-3112), at the Department of Medicine, University of South Carolina School of Medicine, Administration Building, Columbia, SC 29208.

For a registration packet contact Mr. Robert A. Schmidt, Business Manager (217/356-3182), American Society of Andrology, 309 West Clark Street, Champaign, IL 61820.
