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The determination of size and concentration of human sperm with an electronic particle counter

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Two hundred and twenty-three seminal fluid specimens taken from humans were counted both by an electronic particle counter and by the conventional hemocytometer method and the results were compared. An analysis of variance showed that the slope did not differ significantly from unity and the intercept did not differ from zero, which demonstrates a high degree of correlation between the two methods. Sperm concentration was obtained with the model B Coulter counter and with the use of a new diluent. Integral to the technique was the establishment of the upper and lower borders of the sperm population which was done by determining the size distribution pattern for each specimen. The average volume of human sperm has been redetermined and found to range from 10 to 22 μ^3 with a mode of 15 μ^3. The data indicate that the electronic counter is a reliable and practical means for determining the sperm concentration in human seminal fluid. Furthermore, it permits the rapid determination of large numbers of specimens with ease.

Seminal fluid examination is an important part in any investigation of testicular function. The parameters most commonly analyzed are seminal fluid volume, sperm concentration, morphology, and motility. While sperm concentration has been determined by the hemocytometer method for many years, there are a number of limitations inherent in this procedure which have been pointed out but not generally appreciated.[1, 2] These limitations include unequal distribution of particles in the counting chamber and variability in the counting and identification of sperm by the examiner. Also, the problem of technician

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fatigue needs to be considered, especially when a large number of specimens have to be examined. In our laboratory, 200 paired sperm counts performed by two technicians using the hemocytometer method resulted in a deviation of greater than ± 10 per cent of the mean in 20 per cent of the specimens. This large variance in counting also exists with the hemocytometer method when applied to red blood cells.[3]

An electronic counter designed to measure differences in conductivity between particles and surrounding liquid[3] has been used satisfactorily in counting objects such as red and white blood cells and platelets.[2, 4, 5, 6] Segal and Laurence[7] adapted this machine so that human sperm cell volume and concentration could be determined. Unfortunately, there was a wide range of error between the results they obtained for hemocytometer and electronic counting. On the other hand, Glover and Phipps[8] reported a better correlation in their preliminary studies with bull sperm. Since the electronic counter would improve the evaluation of large numbers of seminal fluid samples by virtue of its rapidity, we reinvestigated the problem of its accuracy.

This paper describes an improved method of applying the use of the electronic particle counter to the determination of the size and concentration of human sperm.

Materials and methods

Seminal fluid specimens, collected into clean medicine glasses, were obtained from inmate volunteers at the Washington State Penitentiary. For this study, 223 such specimens were obtained. Following measurement of volume and estimation of motility, the seminal fluid was transferred to shell vials, frozen, and shipped to our laboratory in insulated containers.

Before counting, the seminal fluid was thawed, thoroughly mixed, and 0.1 ml. was diluted (1:1,000) with 100 ml. of a diluent composed of 10 ml. of 1 per cent saponin in isotonic saline, 85 ml. of isotonic saline,* and 5 ml. of glacial acetic acid. Counts were performed 60 ± 15 minutes after dilution since crystalline matter, other debris, white blood cells, and clumping were minimal at this time interval. Severe clumping was occasionally seen with the microscope at 60 minutes. If such was the case, comparative studies were not carried out. If the sperm concentration was less than 10 million per milliliter, the count was redetermined with a 1:500 dilution, if between 200 and 400 million per milliliter, the dilution was increased to 1:2,000, and if the concentration was greater than 400 million per milliliter, a 1:4,000 dilution was utilized.

The electronic counter used was a model B Coulter counter with a 50 μ aperture tube; the aperture current was set at ½ and the amplification at 1. With the use of a 50 λ mercury manometer, 0.05 ml. of diluted seminal fluid was passed through the orifice. The instrument was calibrated with particles of a known volume, i.e., ragweed pollen with a mean corpuscular volume (MCV) of 3,884 μ^3 and human red blood cells, MCV of 86 μ^3.

A particle size distribution plotter was aligned to the upper and lower thresholds of the model B counter. The scale factor, which regulates the height of the graph, was adjusted between 4 and 6 to best achieve the lower point, ("trough"), between the debris and sperm populations, as well as to define the mode of the curve. Four seconds was used as the time required to determine the particle concentration of each window (cycle time).

Total counts were obtained in the following manner: The "trough" between the debris and sperm population was localized and used as the lower border of the sperm population for each seminal fluid specimen. The upper border was determined by finding the point at which the sperm concentration most clearly matched the "trough" in height (Fig. 1). These

*Obtained from Cutter Laboratories, Berkeley, Calif.

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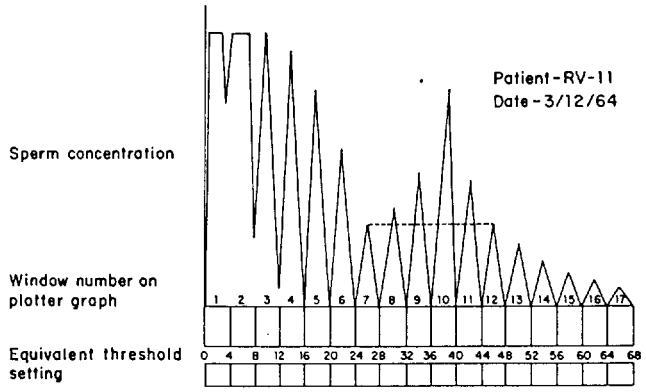

Fig. 1. Diagrammatic representation of sperm size distribution by plotter graph. This is one example of a sperm size distribution obtained. As is indicated in Table II, the curve is usually skewed to the right. However, the upper threshold is determined in the same manner for all curves, regardless of shape.

Table I. Example of estimation of sperm concentration by electronic counter, utilizing plotter graph in Fig. 1

Example	*Window*	*Threshold*	*Estimation*
Lower border of sperm population	7	24	
Upper border of sperm population	12	48	
Uncorrected count between thresholds 24 and 48			1,140
Coincidence factor equals 0.4% or			+ 5
Coincidence corrected count			1,145
Diluent blank at thresholds 24 and 48			− 31
Corrected count minus diluent blank			1,114
Multiplication factor (1:1,00 dilution and 0.05 ml. aliquot counted)			× 20,000
			22,280,000
Electronic count	22 million sperm/ml. seminal fluid		
Hemocytometer count	21 million sperm/ml. seminal fluid		

Patient, RV-11.
Date, 3-12-64.

Table II. Size distribution range for sperm in 100 seminal fluid specimens

	Window on plotter graph with equivalent					
	5 (16-20)	6 (20-24)	7 (24-28)	8 (28-32)	9 (32-36)	10 (36-40)
Lower threshold	7	14	40†	31	5	2
Mode				2	10	61†
Upper threshold						3

*Multiply by $0.4\mu^3$ to obtain volume.
†Mean.
‡Specimens analyzed for mode, 93.

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points are designated on the plotter graph as window numbers. Each window corresponds to 4 threshold divisions on the model B counter. The upper and lower thresholds on the electronic counter were adjusted to approximate the borders of the sperm population determined by the plotter graph. All particles between these threshold settings were determined five times with the model B counter, the plotter being disconnected. The average number of particles was expressed as the uncorrected count. Since the passage of two or more particles through the aperture simultaneously would be registered as a single particle, it was necessary to correct for this phenomenon. The frequency with which this occurs is directly related to the concentration of particles.[2] This has been designated the coincidence factor and was used to adjust the uncorrected count[9] (Table I). To correct for particle contamination of the reagents and glassware, a blank solution comprised of diluent alone was counted five times at the same threshold settings. The mean value thus obtained was subtracted from the coincidence-corrected count. Then the dilution factors were taken into account to obtain the final concentration of sperm per milliliter (Table I).

The electronic and hemocytometer procedures were performed independently by separate technicians. Two to 4 hemocytometer counts were determined and the mean was accepted as the hemocytometer count.

The hemocytometer count was performed initially using a standard red blood cell pipette (1:200 dilution). The same diluent and time factor after dilution were used for both methods of counting. Sperm counts under 20 million per milliliter were recounted using a white blood cell pipette (1:20 dilution). Only intact sperm, heads with attached middle pieces or middle pieces with attached tails, were counted, since the differentiation of the isolated sperm head from debris was considered to be too variable. The results were expressed in millions of sperm per milliliter of seminal fluid.

The reproducibility of the hemocytometer and electronic methods was compared by counting two seminal fluid specimens eight times by each method. Fresh dilutions were prepared for each determination. The results were then analyzed for variance and validity.

Results

The values obtained by calibrating the electronic counter with red blood cells and ragweed pollen were in close agreement, 0.38 μ^3 and 0.39 μ^3 per threshold division, respectively; therefore, each threshold division measured a particle volume of 0.4 μ^3.

The size range of sperm present in 100 seminal fluid specimens is depicted in Table II. The mean values for the lower and upper borders of the population were 24 (range 16-40) and 56 (range 40-68), respectively. These threshold settings represented a mean population range from 10 to 22 μ^3, since each

threshold divisions (in parentheses below)*							*Total No. of specimens*
11 (40-44)	12 (44-48)	13 (48-52)	14 (52-56)	15 (56-60)	16 (60-64)	17 (64-68)	
1							100
19	1						93‡
6	15	21	20†	20	12	3	100

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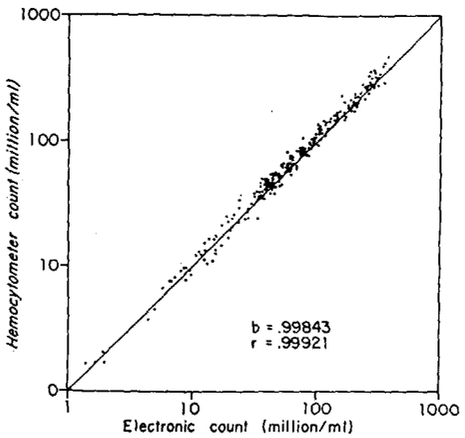

Fig. 2. Comparison of sperm concentration determined by electronic and hemocytometer methods.

Table III. Analysis of variance for slope plotted in Fig. 2

Source	*Degrees of freedom*	*Sum of squares*	*Mean square*
Slope (b)	1	53.48,334	53.48,334
Error	221	0.71,226	0.00,322
Total	222	54.19,560	

Table IV. Analysis of variance for repeated counting of 2 seminal fluid samples by hemocytometer and electronic methods*

	Sperm concentration (million/ml.)		
	Hemocytometer procedure	*Electronic procedure*	*Test for t*
Specimen A (low sperm concentration)			
Mean ($\bar{x}$)	13.92	13.61	Not significant
Variance (S^2)	1.210	0.432	Not significant
Specimen B (medium sperm concentration)			
Mean (x)	83.2	81.9	Not significant
Variance (S^2)	52.19	2.203	< 0.001

*Eight determinations by each procedure.

threshold division measured a particle volume of 0.4 μ^3. The mode of this population was 15 μ^3 and remained constant with varying sperm concentrations.

The sperm concentration for the 223 seminal fluid specimens in this study ranged from 1.4 to 471 million per milliliter. The values obtained by the two methods were plotted against each other in Fig. 2 and analyzed for variance in Table III. The calculated slope was 0.99843 with a correlation coefficient of 0.99921.

Two seminal fluid specimens were counted 8 times by both procedures to determine the variability of each procedure. While there was no statistically

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significant difference in counting the specimen with a low sperm concentration, this was not true of the specimen with a medium sperm concentration. The variance for the hemocytometer and electronic methods was 52.19 and 2.203, respectively, the electronic procedure being significantly less variable (Table IV).

Discussion

When comparing a new technique for estimating sperm concentration with an established method, two questions need to be answered: Do both techniques give similar estimates? Which procedure has less inherent variability?

A calculation of the comparison of 223 paired sperm counts, determined by each of the methods, resulted in a slope of 0.99843 and a correlation coefficient of 0.99921. Since the slope does not significantly differ from 1 ($t = -0.203$) and the intercept does not vary from zero ($t = 0.418$), the sperm counts performed by these two techniques are interchangeable under the conditions we employed.

Although only two seminal fluid specimens were subjected to repeat counting by each method, it appears that the hemocytometer count will be less reproducible under certain conditions. Further study would be necessary before all of the factors are understood.

Analysis of the data of Laurence and Carpuk[10] for 16 sperm counts on guinea pig seminal fluid determined by the electronic method revealed a poor correlation with the hemocytometer technique. Segal and Laurence[7] reported better results using human seminal fluid by grossly grouping their counts into high, medium, and low ranges. However, when one compares the actual numerical values obtained, the agreement between the electronic and hemocytometer methods is not satisfactory. The closer correlation obtained in our study appears to be due to two factors, the use of an improved diluent to effectively remove interfering debris, and the use of variable threshold settings to establish the size range for each individual sperm population.

A variety of diluents have been used for sperm counts to reduce debris and clumping. We had no success with isotonic saline alone,[8] or saponin as a 0.01 per cent solution,[7] and only moderate success with saponin as a 0.1 per cent solution in removing debris. The latter concentration of saponin seemed to prevent clumping, except on rare occasions. The addition of 5 per cent glacial acetic acid to the saponin-saline mixture removed most of the interfering particulate matter without grossly altering the sperm, and thus permitted a sharper discrimination of the sperm population.

Laurence and Carpuk[10] and Segal and Laurence[7] used fixed threshold settings for all samples counted. We found that the boundaries of the sperm population are highly variable in different seminal fluid specimens (Table II). It is evident that identical threshold settings might include excessive quantities of debris or sacrifice part of the sperm population being counted; therefore, a graphic representation of size distribution is necessary on each specimen to determine the borders of the sperm population. The total count may then be obtained between these boundaries utilizing the electronic particle counter.

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With counts below 10 million per milliliter, the boundaries of the sperm population become increasingly difficult to define on the plotter graph. By increasing the concentration of particles twofold (1:500 dilution) from the standard dilution, one obtains increased resolution of the sperm population. Sperm concentrations above 200 million per milliliter of seminal fluid exceed the capacity of the electronic counter. In these instances, dilutions greater than 1:1,000 produce concentrations within a range suitable for counting by the machine.

The mean volume range for human sperm presented in this paper (10 to 22 μ^3) is at variance with the results of Segal and Laurence[7] (28.0 to 78.4 μ^3). Since their graphic representation of the sperm population is similar to our findings, the disagreement must be in the calibration of the electronic counter. Our calibration was performed with two different types of particles (ragweed pollen and red blood cells) with close agreement. Dr. K. A. Laurence has informed us that an error was discovered in his and Dr. Segal's calibration. We understand that a report to this effect is being prepared.

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