PRELIMINARY STUDY OF AN ELECTRONIC METHOD OF COUNTING AND SIZING BULL SPERMATOZOA

F. A. GLOVER AND L. W. PHIPPS

National Institute for Research in Dairying, University of Reading

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Summary. An electronic particle-size analyser has been used to examine the size distribution of spermatozoa in bull semen. A complete size distribution may be obtained on 10,000 spermatozoa in about an hour. Determinations of the concentration of spermatozoa in semen made with the instrument were found to be highly correlated ($r = 0.96$) with corresponding estimates obtained from direct counts with the microscope.

INTRODUCTION

The most useful guide to the quality of a sample of semen is the number of spermatozoa per millilitre. Normal concentrations of spermatozoa in bull semen are from 1 to 30×10^8 per ml, with an average of about 10×10^8 per ml. Estimates of the concentration are commonly made with the microscope from the number counted in a known volume of the diluted semen. The method is tedious, however, and liable to quite large sampling and subjective errors. According to Bane (1953), the total error expected in a single determination is roughly 10% of the true number, about half of the error arising from the filling of the chamber and the counting, and the remainder from heterogeneity of the undiluted semen. A quicker estimate of the concentration may be made indirectly by measuring the optical density of diluted semen with a photo-electric absorptiometer calibrated against the microscope. Cox & Melrose (1953) have shown that a linear relation holds and that the optical method is adequate for requirements of artificial insemination. In a recent paper, Glover & Scott Blair (1961) found that the total sperm count measured with the absorptiometer was highly correlated ($r = 0.92$) with the rise in apparent viscosity (measured at 50°C) of the semen which occurs when it is heated for 60 min, as compared with 10 min, at 50°C . Although this viscosity method for estimating sperm concentration is unlikely to be of much practical value in the form described, a modification of it together with improved standards of comparison might lead to a useful empirical test. The fairly recent development of an electronic particle counter affords a further direct method for determining spermatozoa concentrations with possible use as a standard of comparison. The main advantage of the method is that very large numbers of particles can be counted automatically in a very short time. This paper describes the application of the electronic Coulter Counter (Coulter, 1956) to counting and sizing bull spermatozoa.

Since completing the experiments described in this paper Dr S. J. Segal has kindly informed us of a paper which is shortly to appear describing similar work on human spermatozoa (Segal & Laurence, 1962).

METHODS

The apparatus is described in detail by Mattern, Brackett & Olson (1957). Briefly, the particles to be examined are diluted with an appropriate electrolyte and the suspension made to flow through a circular aperture, in this work 30 to 100 μ in diameter, across which a voltage is maintained. As a particle passes through the aperture, an abrupt change in the electrical resistance occurs which is proportional to the particle volume. The resulting voltage pulse is amplified and may be counted electronically. The response of the instrument to particle volume may be adjusted so that, for a given setting or threshold level, only particles larger than a certain size are counted. By taking a succession of counts in equal volumes of suspension at different thresholds, a cumulative size distribution curve may be obtained. The instrument is conveniently calibrated with a suspension of spherical particles of narrow, accurately known, size range. Suspensions of ragweed pollen (about 20 μ in diameter) and polystyrene spheres (about 1 μ) are among those suitable for this purpose. 'Coincidence counts', that is the passage through the aperture of more than one particle at a time, can lead to total counts which are less than the true values. However, by adequate dilution, the effect is suitably limited and can be allowed for in a standard way in the analysis of the data. The aperture diameter to be used depends upon the size range of the particles to be counted. As a useful working rule, the particle sizes should lie within 4 to 40% of the aperture diameter.

The unique, asymmetric shape of spermatozoa raises a number of questions, in particular the best aperture size to use and the nature of the response of the instrument, especially the extent of coincidence losses. The dimensions of the bull spermatozoon have been given by Bretherton & Rothschild (1961); the head, shaped like an elliptical disc, is 8.5 to 10 μ long, 4.5 μ wide and 0.7 μ thick: the middle piece is 15 μ long and 0.8 μ in diameter; the tail, 45 μ long and 0.3 μ in diameter. The diameter of the sphere of equivalent average volume is thus calculated to be about 4.0 μ . On this basis, and allowing for some spread of the sizes, the available standard aperture diameters of 30 and 50 μ would be considered suitable, with a preference for the larger size for ease of working and greater freedom from adventitious blocking by foreign matter during operation. Preliminary experiments confirmed that dilute suspensions of spermatozoa flowed freely through a 50 μ diameter aperture and this size was used throughout the present experiments. In connexion with the flow behaviour, it seems probable that the spermatozoa are orientated in the fluid velocity gradient upon approaching the aperture and that, when passing through, their lengths are aligned parallel to the streamlines (Bretherton & Rothschild, 1961).

Experiments at a number of different dilutions, together with comparisons against the microscope method, led to the conclusion that no undue bias towards high coincidence counts occurred when counting spermatozoa. A high rate of coincidence counts would tend to lead to low estimates of concentration.

Anticipating a result from the next section, estimates of concentrations from electronic analyses were generally found to be a few per cent higher than those from the microscope. The reasons for this are not clear, but multiple counting might occur when spermatozoa are unfavourably orientated and touch the aperture walls or, indeed, the instrument may be recording true concentrations. In the latter case, the microscope counts are low.

The diluent used in the present experiments was normal saline solution (B.P., 0.9% w/v) as prepared commercially for injection purposes. Although this was mainly free from dust particles, routine counts were taken on the solution at the appropriate threshold levels before each experiment so that their contribution to the total count could be subtracted. The volume of the spermatozoa suspension automatically metered by the instrument was chosen to be 50 μ l throughout.

For the microscope technique, dilutions of 1 : 30 were mainly used, the suspension being mounted on a haemocytometer slide. The number of spermatozoa in six sets of sixteen squares, each square representing a volume of 1/4000th mm^3 were counted, i.e. about 1000 spermatozoa.

RESULTS AND DISCUSSION

Text-fig. 1 shows a typical derivative size distribution curve obtained with the electronic analyser of the particles in a sample of bull semen taken from a normal animal used for artificial insemination. The rate of dilution with saline solution was 1 : 6000 carried out with careful mixing in two stages, 1 : 60 followed by 1 : 100. The total time required for the analysis was about an hour.

It may be seen that most of the particles occur in the size range 2 to 4 μ

TEXT-FIG. 1. Typical size distribution of the particles in bull semen. Dilution 1 : 6000.

within which the distribution is approximately Gaussian. The curve has a mode of about 3.2 μ which corresponds fairly closely with the diameter 4.0 μ , calculated above, of the sphere having the same volume as a bull spermatozoon. It seems reasonable, therefore, to regard the part of the curve to the right of the minimum that occurs at about 2.0 μ as representing numbers of spermatozoa and that to the left to be due to other smaller cells, sperm fragments and dust. It follows that, with due regard to background and coincidence loss corrections, the total count recorded at a threshold level equivalent to the minimum in the

distribution curve is the number of spermatozoa in 50 μ l of suspension. Knowing the dilution factor, the concentration of the natural semen may be calculated. It should be noted that spermatozoa heads alone as well as complete tail pieces have volumes whose equivalent sphere diameters, approximately 3.7 and 2.8 μ , respectively, are large enough for them to be mistakenly counted as whole spermatozoa. It follows that for abnormal semen containing sperm fragments of this size, the determination of the concentration would tend to be too high.

In experiments to examine the effect of the rate of dilution upon the results, there was a suggestion of closer agreement with the microscope counts the greater the dilution. However, in a typical series with eight different semen samples at the three dilutions 1 : 3000, 1 : 6000 and 1 : 12000, an analysis of variance showed the trend to be statistically quite insignificant. It was finally concluded that a suitable dilution for this work was between 1 : 3000 and 1 : 6000. Further analysis showed that the total variance of replicate estimates of the concentration of one semen was about 0.3×10^{16} , the greater proportion

TABLE 1
SUMMARY OF CORRELATION RESULTS

Correlation coefficient	0.96	
Regression equations	$M = 0.9 + 0.89C$ $C = 1.03M$	
Residual s.d. about the regression line ($\times 10^{-8}$)	C	M
	1.1	1.0
5% Fiducial limits ($\times 10^{-8}$)	± 2.5	± 2.3
Range of semen concentrations	6 to 20×10^8 /ml	

M: microscope estimate of semen concentration per ml $\times 10^{-8}$
C: electronic estimate of semen concentration per ml $\times 10^{-8}$

of this arising from the sampling procedure, the remainder (about 2%) being due to variation in the repeatability of the instrument count.

In order to calculate the correlation between the microscope and electronic methods of estimating sperm concentrations, experiments were carried out on fifteen different semen samples. The sperm concentrations estimated from the counts obtained at the minima of the size distribution curves were found to be highly correlated with the microscope estimates, the correlation coefficient being 0.96. The equations to the two regression lines relating the microscope to the electronic values and vice versa have been determined and are shown in Table 1. In addition, the standard deviations of the scatter about the lines are given as well as the 5% fiducial limits, that is, the limits on either side of the regression line within which 95% of the points lie.

It was observed in the experiments that the minima in the distribution curves from the electronic analyses occurred at threshold levels corresponding to sizes close to 2.2 μ . Accordingly, the sperm concentrations estimated from the

counts obtained at this one setting were also compared with the corresponding microscope estimates. The correlation was found to be almost as good as before with a coefficient of 0.95 and the regression lines and other data in close agreement with those in Table 1.

Although the results of these experiments show that there is a high degree of correlation between the two methods, it is open to question which one should be regarded as providing the truer count. The electronic method tends to give results which are about 3 to 4% higher than the microscope estimates and possible reasons for this have been mentioned earlier. The data suggest that, to improve the precision of the measurements, attention should be paid to the sampling procedure since most of the variability arises from this source. The time taken in diluting, counting at a single threshold level and calculating the sperm concentration has been found to be approximately 20 min. Obtaining the concentrations from the minimum in the distribution curve requires very little more time, since counts at only three or four threshold settings are needed to locate the minimum. The time for an estimate with the microscope is 30 to 40 min. The electronic method, therefore, would possess advantages over the microscope technique when concentrations of a number of semen samples were required to be known quickly. Nevertheless, it would probably be slower than the absorptiometer method, although the relative accuracies of the two methods might be a consideration in this case.

In this preliminary study, the potentialities of the electronic instrument have not been fully exploited, but undoubtedly its superiority lies in the high speed of counting and the ease with which a complete size analysis can be carried out. Furthermore, although the instrument provides no information on particle shape, it could be expected to have decided advantages over other methods in examining the morphology of semen, a factor which, as well as sperm concentration, is important in assessing quality.

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Note added in proof

We have always appreciated that the size distribution obtained with the Coulter Counter is considerably wider than it appears to be from observations with the microscope. In view of a recent publication by Kubitschek (*Rev. Sci. Instrum.* **33**, 576, 1962), this broadening could be an instrumental effect arising out of the relative lengths of the spermatozoon and its path through the aperture.