

The estimation of sperm motility in semen, on a membrane slide, by measuring the area change frequency with an image analysing computer

H. M. Dott and G. C. A. Foster

A.R.C. Institute of Animal Physiology, Animal Research Station, 307 Huntingdon Road, Cambridge CB3 0JQ, U.K.

Summary. A simple slide is described in which the base is a permeable membrane so that a suspension of spermatozoa (or other cells) may be examined under controlled conditions with a microscope. An objective method of assessing the motility of spermatozoa in undiluted or diluted semen from a wide variety of species including cattle, horse, pig, rabbit, rat and sheep is described. It is shown to be well correlated with other methods of assessing motility and also with the non-return rate obtained with frozen cattle semen.

Introduction

The motility of a population of spermatozoa is an important criterion for assessing the quality of the population both in practical terms, before artificial insemination, and in the laboratory for determining the effect of experimental procedures.

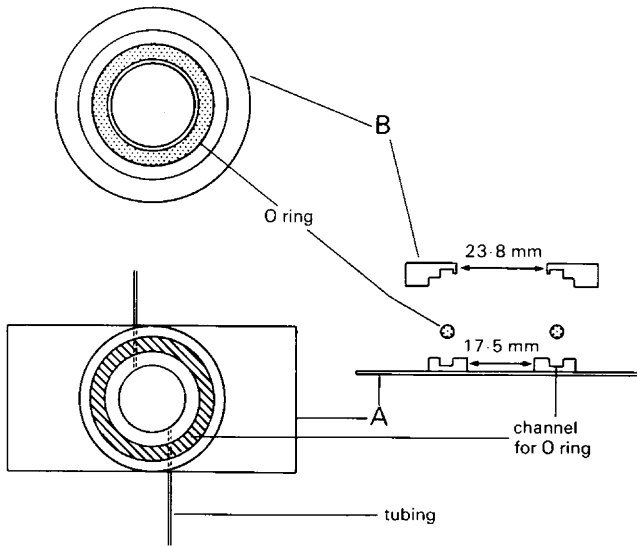
The objective, quantitative methods for assessing sperm motility are reasonably accurate and repeatable but each of them has a limited application; those which are based on the mass movement of spermatozoa (e.g. Rothschild, 1948; Walton, 1952; Glover, 1968) require a suspension with a high sperm density in which a high proportion of the spermatozoa are active, and methods for measuring the proportion of motile spermatozoa and the mean velocity require a suspension diluted in such a way that individual spermatozoa can be observed (Harvey, 1945; Rothschild, 1953; Rikmenspoel, 1957; Dott, 1975; Katz & Dott, 1975). The results obtained with all these methods correlated well with subjective estimates made by an experienced observer reading samples on a scale appropriate to the type of semen being examined. Since the scale is chosen in this way, subjective estimates have a wider range than any one of the objective methods but the results are essentially descriptive and liable to vary from time to time and from observer to observer. In this paper we describe a method for obtaining an objective assessment of motility which can be applied to semen or diluted semen and in this sense is as wide ranging as the subjective method.

Materials and Methods

Membrane slide (see Text-fig. 1)

In order to provide the spermatozoa with an environment in which there was a large enough volume of sample to permit random movement of the spermatozoa in all directions with a sufficient oxygen supply to maintain motility, a special slide with an oxygen-permeable base was constructed (Miles & Dott, 1979).

The slide was made of Perspex with two stainless-steel tubes going into the well below the sample chamber. To make the sample chamber a piece of gas-permeable membrane (Vitafilm



Text-fig. 1 Scale diagram of the support for the semipermeable membrane of a membrane slide. The membrane is placed over the Perspex supporting ring (A) and held in place by the securing ring (B).

F10 GP, Goodyear Tyre & Rubber Co., Gt. Britain Ltd), approximately 50×50 mm was placed on a clean flat surface and any wrinkles smoothed out. A self-adhesive plastic reinforcing washer was stuck onto the centre of the membrane. This produced a membrane-based sample chamber with a depth of about $120 \mu\text{m}$ (single chamber). To produce a chamber of greater depth a second washer was sometimes placed over the first, giving a sample chamber with a depth of about $240 \mu\text{m}$ (double chamber). The membrane was then positioned on top of the Perspex support ring (A). The Perspex securing ring (B) containing the O ring, was then forced down onto the support ring so that the plastic membrane was tensioned and locked into place with the sample chamber directly above the well of the slide. This produced an optically flat surface as the base of the sample chamber.

When a sample of semen was introduced into the sample chamber, the volume used was sufficient to fill the chamber when a 22 mm diameter coverslip (No. $1\frac{1}{2}$ Chance) was placed on top.

Measurement of area change frequency (ACF)

The method depends on the ability of an image analysing computer (Quantimet 720: Cambridge Instrument Co., Melbourn, Herts., U.K.) to detect and measure areas of equal brightness (grey levels) in the image of a semen sample produced by a microscope. The number of times the measured area changes during a period of 30 sec (area change frequency, ACF) is dependent on the motility of the spermatozoa in the sample.

The sample of semen was placed under the microscope and the image transferred to the screen of the Quantimet. After preliminary experiments in which various modes of operation were compared it was decided to adopt a 'slice detection' mode, i.e. the area between selected grey levels, to measure area and to have a maximum size. Other modes measure different mean areas but give the same ACF suggesting that the setting of the machine is not critical.

The area occupied by the selected grey level was measured 180 times (6/sec for 30 sec). The measurements were stored in a desk top calculator (Wang 600) until all 180 measurements had

been completed, when they were examined to see if consecutive areas differed from each other. (The number of measurements and rate of collection were governed by the capacity of the equipment.) The area change frequency (ACF) was obtained by counting the number of times the area increased and decreased. As there was always some change in consecutive measurement of area (due to background noise) a minimum change of 1000 picture points (a picture point is the minimum measure of area on the screen, the total measurable area being 5×10^5 picture points) was required to establish an area change count. As the area was measured 180 times the theoretical maximum ACF was 179/30 sec.

A second run on the same sample was carried out immediately and the two ACFs obtained were tested for compatibility. To be compatible the counts could not differ from their mean by more than the square root of the mean. If they were not compatible a third ACF determination was performed and compared with each of the first two counts for compatibility. When two compatible counts were obtained the mean was printed out and it was regarded as the ACF of the sample. In the event of none of the counts agreeing no overall ACF figure was printed, and a fresh sample was measured (this only occurred in 2 out of >1000 measurements). The time taken for measurement and calculation for 2 readings is 1 min 20 sec and for 3 readings 1 min 53 sec.

The depth of chamber, single or double, and the type of microscopic examination used were changed according to the type of semen being investigated.

Semen

Samples of semen from different species and of differing concentrations and degrees of motility were used; their ACFs were determined and when possible compared with other motility estimates of the sample.

Samples of ejaculated semen were obtained from bull, rabbit, ram and stallion by means of an appropriate artificial vagina. Samples of rabbit and ram epididymal spermatozoa were obtained by flushing out the epididymis (Dott, Harrison & Foster, 1979). Samples of frozen bull semen were obtained from Cambridge Cattle Breeders.

ACF determination for semen samples showing wave motion (Method 1)

The semen sample was placed in a sample chamber of sufficient depth (single or double) to permit the wave motion of the sample to become established. The sample was placed under the microscope and examined with phase-contrast illumination and a $\times 10$ objective. The image was then transferred to the Quantimet screen and the slice detection adjusted so that the boundaries of the waves were detected, the alteration of the detected area being caused by the change in size and shape of the waves.

ACF determination for semen samples of insufficient sperm density to show wave motion (Method 2)

The semen sample was placed in a single sample chamber and examined with phase-contrast illumination and a $\times 10$ objective. The image was then transferred to the Quantimet screen and the detection was set so that it corresponded with the area which was altered in size and shape by the movement of the spermatozoa. The sensitivity was adjusted so that a mean count of approximately 40 000–60 000 picture points was obtained. To obtain a change in regions of equal slice detection in some species it was necessary to use dark-ground illumination. In these samples the changes in area were caused in part by the movement of the spermatozoa in and out of the focal plane and partly by overlapping of spermatozoa.

In both methods the measurements were made between 20 and 22°C.

Experimental tests

Comparison of ACF with other methods of assessing motility

Two types of semen have been used: (a) semen displaying wave motion, i.e. ejaculates of bull and ram, and (b) frozen semen from bulls being used in the A.I. programme of the Cambridge Cattle Breeding Centre.

Semen displaying wave motion. A single-depth chamber was used for ram semen and a double-depth chamber for bull semen. The impedance change frequency (ICF; Rothschild, 1948) was measured on samples taken at the same time as the samples for ACF. The range of ICF in 21 samples of bull semen was 0–56 changes/min and of ACF 0–38 changes/30 sec (the correlation coefficient was 0.84, 19 d.f., $P < 0.001$). The range of ICF in 89 samples of ram semen was 0–153 changes/min and of ACF 0–74 changes/30 sec (correlation coefficient 0.88, 87 d.f., $P < 0.001$). The first two readings were compatible for 9/10 readings; the need for a third reading was usually due to the presence of large foreign bodies, such as an air bubble in the sample chamber.

Frozen bull semen. Two 0.25-ml straws of frozen semen were obtained from each of 3 separate ejaculates of 5 different bulls. Before freezing the semen had been diluted with an egg yolk–glycerol diluent so that the concentration of spermatozoa was approximately the same in all straws (1×10^8 spermatozoa/ml). Samples of semen from each of the bulls taken over a period of at least 1 year had been assessed by eye by an experienced observer who ranked the bulls in order for (a) the quality of semen produced and (b) the fertility of the bull (see Table 1).

Each straw was thawed by immersion in a water bath at 30°C for 10 sec. The straw was then emptied into a small glass tube and the contents incubated at 30°C. After 2 min a 10 μ l sample of the semen was placed in a single chamber on the membrane slide, covered and put under the microscope at 22°C. Determinations of ACF were carried out at 2 min intervals until either an ACF of zero was obtained or until four determinations had been made (Time 0). At 1 h after thawing (Time 1) a fresh sample of the thawed semen was taken and ACFs of this sample were determined, again at 2 min intervals, until either a zero reading was obtained or 4 determinations had been made.

The regressions of ACF on time were calculated for the four readings or until a zero ACF had been given whichever was the shorter time. If the ACF declined (i.e. the regression coefficient had a probability of <0.01 of being due to chance, 6 out of 60) then the intercept (i.e. ACF at zero time) was regarded as the ACF of the sample; for samples in which the ACF did not decline (i.e. the regression coefficient was not significant) the mean of the 4 ACFs was taken as the ACF of the sample. (In some samples readings continued for 18 min but the extra readings gave no additional information and results calculated from them did not differ significantly from the first 4 readings.)

For each bull there was no significant difference between ejaculates at Time 0 or at Time 1. The three ejaculates were therefore grouped together for each bull for Time 0 and Time 1 to provide 6 replicates for each time. Bulls A and E had higher ACF values than Bulls B and C which had higher ACF values than Bull D ($P < 0.001$, d.f. 4, 50). All bulls had lower ACF values at Time 1 than at Time 0 and in Bulls B and E this was statistically significant ($P < 0.02$, d.f. 10 for Bull B and 7 for Bull E).

A visual assessment of sperm motility was made at Time 0 and Time 1 prior to the initial ACF determination in each run. The motility was scored on a scale of 0 to 5 with 0 being immotile and 5 having excellent motility. There was a strong correlation between visual assessment and ACF value ($r = 0.93$, $P < 0.001$, d.f. 8). A single figure representation for the semen samples of each bull was calculated by subtracting the difference between ACF at Time 1 and Time 0 from the ACF value at Time 1 and was called the motility survival index (MSI): the results are given in Table 1. A high MSI can only be achieved by a sample of semen in which the

spermatozoa have good initial motility and good survival of motility. If either of these is poor a low MSI will be obtained.

Comparison with non-return rate

Conception rates for each bull were known and shown by a χ^2 test to be significantly different ($\chi^2 = 10.05$, $P < 0.01$, d.f. 4), with Bull A having the highest non-return rate and Bulls D and E the lowest.

There was a correlation of 0.63 between MSI and non-return rate ($P < 0.01$, d.f. 120). The two bulls (A and E) giving the highest ACF value at Time 0 were also graded as the best semen producers, B and C were rated as the next best bulls while Bull D was the worst both by reference to ACF value and to the rankings (Table 1). The low ranking of Bull E with regard to fertility also corresponded with its significant decline in ACF between Time 0 and 1 and its low MSI. Although Bull D had a low ACF at both Times 0 and 1 it did not decline significantly between those times. Bull C did not have as high an ACF value as Bull E but the value did not decrease with time. In fact Bull C had a higher MSI than Bull E and than Bull B which was next in rank below it for fertility.

During this experiment 620 ACF values were determined. The first two readings were not compatible in only 72 cases for all of which a third reading was obtained.

Table 1. Estimates of motility and fertility of frozen-thawed bull semen

Bull	No. of samples	Total inseminations	% non-return by 60 days	Fertility ranking	ACF			Visual assessment		Semen ranking
					Time 0	Time 1	MSI	Time 0	Time 1	
A	6	257	86	1	20.06	16.77	+13.48	3.58	3.25	1
B	6	223	74	3	10.78	4.35	-2.08	2.9	1.75	4
C	6	296	78	2	10.4	5.67	+0.94	2.9	2	3
D	6	250	67	5	2.65	0.71	-1.23	1.8	0.9	5
E	6	422	67	4	23.36		-4.56	3.5	2.75	2

Other types of semen

The ACF can be used to assess motility in any type of semen and probably on many types of cells moving in a random fashion. We have measured ACF in ejaculates of the stallion, boar and rabbit, and 2 experiments have been done with rat epididymal semen (Ford & Waites, 1978; Hinton, Dott & Setchell, 1979). We have not used alternative methods with the samples from these species; however, subjective estimates agreed with the ACF values in that samples in which the motility was poor gave a low ACF. Determinations of ACF values in different samples of semen are not comparable if the spermatozoa in those samples have dissimilar types of motility, e.g. an ACF derived from wave motion (Method 1) can not be compared with an ACF when wave motion is absent (Method 2).

The membrane slide

The membrane slide allows a sample of semen to be examined under the microscope under controlled conditions; the nature of the membrane dictates to some extent the conditions which prevail. For the membrane used in our experiments the oxygen tension remained at a sufficient level to maintain the motility of the spermatozoa and evaporation was prevented. As an

illustration of this, some samples of undiluted ram semen have exhibited wave motion for over 6 h on a membrane slide and a sperm-rich fraction of boar semen, which is particularly sensitive to O₂ deprivation (Dott, 1958), exhibited wave motion for up to 90 min. Other membranes could be used, e.g. a dialysis membrane, with a dialysing fluid pumped through the well beneath the sample chamber to supply substrates, maintain pH and remove metabolites. Pumping fluid through the well also enables the temperature to be controlled.

The depth of the sample chamber can be adjusted to suit the type of measurement being made; for determination of ACF values, depths of 120–240 µm proved suitable. For measurement of the proportion of motile spermatozoa (Dott, 1975) or sperm velocity (Katz & Dott, 1975) 20–30 µm would be used.

The ACF appears to be a measure of the activity of spermatozoa; when it is used on samples of semen which display wave motion it correlates well with impedance change frequency (ICF) and it is possible that both measurements refer to the same phenomenon. When ACF is produced by dilute samples of semen it is a composite figure based on the number of moving spermatozoa and the vigour of their motility and because it is a composite figure it does not supply useful information about either. Nevertheless it is an objective measure of a phenomenon which previously was measurable only on a subjective scale.

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