Evaluation of cellulose acetate/nitrate filters for measuring the motility of dog spermatozoa

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Summary. A modified chemotaxis chamber was used to evaluate cellulose acetate/nitrate filters for the measurement of dog sperm motility. The distance travelled into the filters was affected by filter pore size, incubation time and sperm concentration. After storage of spermatozoa at 37°C the distance penetrated into the filters reflected the deterioration of the sample, but was not as sensitive a measure as the mean sperm velocity or the percentage of motile spermatozoa.

Keywords: cellulose acetate filters; spermatozoa; motility; dog

Introduction

The evaluation of sperm motility is an important part of the examination of a semen sample. Emmens (1947) introduced the concept of the motility index to assess both the percentage and the degree of sperm motility. This system is still widely used although many workers have attempted to produce more quantitative systems. Harvey (1960) calculated sperm velocity by timing the movement of spermatozoa across a grid. Other workers have used time-lapse photography (Rothschild, 1953; Makler, 1978; Van Huffel *et al.*, 1985), the scattering of laser light by moving spermatozoa (Ross *et al.*, 1983), computer-assisted measurements (Holt *et al.*, 1985) and computer image analysis (Katz & Dott, 1975; Working & Hurtt, 1977) to evaluate sperm motility. Some workers have suggested that sperm motility is the best predictor of human male infertility (MacLeod & Gold, 1951; Hartman, 1965) although others have questioned this absolute relationship (Katz & Yanagimachi, 1981).

Polycarbonate filters have been used to evaluate the effect of chemotractants and pharmacological agents upon sperm motility (Hong *et al.*, 1981; Gnessi *et al.*, 1985). These studies quantified the numbers of spermatozoa passing through thin filters. Zigmond & Hirsch (1973) described a technique which allowed neutrophils to migrate through a thick filter. The filters were then mounted and examined microscopically. The distance from the top of the filter to the furthest plane of focus which contained 2 cells was measured. Zigmond & Hirsch (1973) stated that this method, the 'leading front' technique, was a sensitive and reproducible indicator of the rate of locomotion. The technique was adapted by Strzemienski *et al.* (1987) to evaluate stallion sperm motility. These workers used cellulose acetate/nitrate filters placed into Sykes–Moore chemotaxis chambers. They concluded that the distance travelled into the filter reflected the motility of the ejaculate.

The present study was conducted to evaluate cellulose acetate/nitrate filters in a modified chamber for the measurement of dog sperm motility.

Materials and Methods

Semen was collected by digital manipulation from 6 healthy beagles of unknown fertility. The three fractions of the ejaculate were collected into three separate test tubes via glass funnels. The second fraction was examined microscopically at 37°C to assess the percentage of motile spermatozoa. The concentration was measured using a

haemocytometer chamber, and vital staining and morphology were examined on nigrosin/eosin stained smears. Semen was collected twice weekly from each dog.

Chamber assay. Sykes–Moore chemotaxis chambers are expensive and cumbersome; a modification was described by Sedgwick (1982) for the evaluation of neutrophil locomotion. Briefly, upper chambers were assembled by cutting 1-ml disposable syringe barrels to a length of 5.5 cm. To the cut surface a 7-mm diameter cellulose acetate filter (SM filter type, Millipore Corporation, Harrow, Middlesex, UK) was attached using a special adhesive (MF Cement, Millipore Corporation). A 5-ml disposable syringe barrel was used as the lower chamber. The rubber portion of the plunger was removed and reinserted to occlude the luer fitting end. Upper chamber length and the volumes of the media and sperm suspension are critical to avoid hydrokinetic forces. Lower chambers were placed upright in a metal rack and 0.9 ml minimal essential medium (Flow Laboratories, Rickmansworth, Herts, UK) at 37°C was added. This medium has been shown to support dog sperm motility at 37°C (G. C. W. England & W. E. Allen, unpublished observations) and is simple to prepare and use, unlike other media (Mahi & Yanagimachi, 1978). The upper chamber was lowered into the medium and 100 µl of semen or semen suspension were added. The chambers were incubated at 37°C for 1 h. Then the filters were fixed overnight in 10% buffered formal saline. They were washed 3 times in tap water, stained for 5 min with haematoxylin, progressively dehydrated by immersion in 70%, 90% and absolute alcohol and then cleared and mounted on glass microscope slides. The distance travelled by the spermatozoa was measured (using the micrometer on the fine focus of the microscope) as that between the top of the filter and the furthest plane of focus which contained at least 2 sperm heads. Six readings were taken for each filter and 3 filters were used for each test sample.

Experiment 1: effect of pore size. Five ejaculates were collected from each of the 6 dogs. The ejaculates were divided and placed into chambers with filters of pore size 3, 5 and 8 µm. The filters were incubated for 1 h.

Experiment 2: effect of incubation time. Five ejaculates were collected from each of the 6 dogs. Chambers were assembled with 5 µm filters on the basis of the results of Exp. 1. The chambers were incubated for 15, 30, 60 or 120 min.

Experiment 3: effect of dilution. Because of the inaccuracies of any method of determining sperm concentration, the dilution of spermatozoa to an absolute concentration is extremely difficult. The concentrations from the 6 dogs used were similar and consistent throughout the study. For this reason semen was diluted volume for volume with minimal essential medium. Five ejaculates were collected from each of the 6 dogs. Samples were diluted to 1:2, 1:4, 1:8, 1:16 and 1:32 of the original concentration; these, and undiluted samples, were placed into chambers (5 µm filter) and incubated for 1 h.

Experiment 4: relationship between distance penetrated and the motility, velocity and morphology of spermatozoa. Four ejaculates were collected from each of the 6 dogs. The semen was diluted to 1:8 of its original concentration for measurement of sperm velocity. Immediately after dilution the samples were placed into chambers (5 µm filters) and incubated for 1 h. Samples were also placed into Helber bacterial cell-counting chambers (Weber Scientific Ltd, Teddington, Middlesex, UK) at 37°C; an assessment was made of the percentage of motile spermatozoa and video images of swimming spermatozoa were recorded for subsequent analysis. Smears were stained with nigrosin-eosin for the evaluation of live spermatozoa and sperm morphology. The remaining diluted samples were kept in stoppered tubes at 37°C, and the procedures described above were repeated at 2, 4 and 6 h after ejaculation. Mean sperm velocities were measured from the video recordings using a semiautomatic computerized technique (Holt *et al.*, 1985).

Experiment 5: penetration by formalinized spermatozoa. One ejaculate from each of the 6 dogs was diluted to 1:8 of its original concentration with 10% buffered formal saline. After 5 min the diluted semen was placed into chambers (5 µm filters) and incubated for 1 h.

Statistical analysis. The mean values were calculated for each treatment group and significance was assessed using the one-way analysis of variance coupled with partitioning and the method of least significant difference. Values were considered significant when $P < 0.05$.

Results

During the study ejaculates from all 6 dogs were within the ranges of 90–95% motility, 300–500 × 10⁶/ml concentration, and 80–92% morphologically normal live spermatozoa.

Experiment 1

The distances travelled by spermatozoa into the three different pore size filters were significantly different ($P < 0.001$). Spermatozoa travelled the least distance into the 3 µm filter (23 ± 7 (s.d.) µm) and the greatest distance into the 8 µm filter (104 ± 17 (s.d.) µm). The spermatozoa travelled approximately half way through the 5 µm filter (47 ± 12 (s.d.) µm). Spermatozoa were frequently noted to have traversed the whole depth of the 8 µm filter.

Experiment 2

The distance travelled by spermatozoa into the filter was greater the longer the incubation time. These differences were significant with the exception of the distance travelled between the samples incubated for 60 and 120 min (Table 1).

Table 1. Relationship between incubation time and mean distance penetrated by dog spermatozoa into cellulose acetate/nitrate filters (5 μ m pore size, $n = 30$)

Incubation time (min)	Sperm penetration (μ m)	
	Mean	s.d.
15	29	10
30	42	12*
60	53	10*
120	56	8

*Mean distances significantly greater than value for previous incubation time ($P < 0.001$).

Experiment 3

Dilutions of 1:2 and 1:4 produced a significant increase ($P < 0.01$) in distance travelled by the spermatozoa; dilutions above 1:8 produced a significant reduction (Table 2). At dilutions of 1:8 there was no difference in penetration when compared to the raw sample.

Table 2. Relationship between sample dilution and mean distance penetrated by dog spermatozoa into cellulose acetate/nitrate filters (5 μ m pore size, $n = 30$)

Dilution	Sperm penetration (μ m)	
	Mean	s.d.
Raw	51	8
1:2	57	11*
1:4	61	9**
1:8	49	11
1:16	39	6**
1:32	30	7**

*Significantly different from raw sample ($P < 0.05$).

**Significantly different from raw sample ($P < 0.01$).

Experiment 4

Storage of the diluted semen samples produced a decrease in the distance travelled, the percentage motility, the mean velocity, and the percentage of live and morphologically normal live spermatozoa. These changes were all statistically significant after storage for 2 h (Table 3).

Table 3. Relationship between various seminal characteristics of dogs at different times post ejaculation (5 μ m pore size, $n = 24$)

	Time after ejaculation			
	Initial	2 h*	4 h*	6 h*
Penetration (μ m)	51 $\pm$ 2	40 $\pm$ 3	31 $\pm$ 4	24 $\pm$ 3
% Motility	94 $\pm$ 2	84 $\pm$ 7	43 $\pm$ 16	12 $\pm$ 12
Velocity (μ m/sec)	> 200 $\pm$ 0	118 $\pm$ 9	66 $\pm$ 12	33 $\pm$ 14
% Total live	90 $\pm$ 4	83 $\pm$ 5	77 $\pm$ 5	62 $\pm$ 9
% Normal live	80 $\pm$ 5	70 $\pm$ 7	59 $\pm$ 5	48 $\pm$ 8

Values are mean $\pm$ s.d.

*All values significantly different from initial sample ($P < 0.05$).

To assess the degree of change in the measured parameters each was expressed as a percentage decrease from the original value (Fig. 1). After incubation for 2 h, the distance travelled, the percentage motility, the percentage live and morphologically normal live spermatozoa fell to 78–92% of their original values. Speeds of greater than 200 μ m/sec could not be measured accurately as they were above the detection limits of the system configuration used. The fall of mean velocity was therefore to at least 58% of its original value. After longer periods of storage there was a large decrease in percentage motility; by 6 h there were many live morphologically normal spermatozoa present, most of which were non-motile.

Fig. 1. Effect of storage at 37°C on various seminal characteristics of dogs (TLS = total live spermatozoa; NLS = normal live spermatozoa; DTF = distance travelled into filter; PM = % motility; MSV = mean sperm velocity). Values are mean $\pm$ s.d., $n = 24$. All values are significantly different ($P < 0.05$) from initial value before storage.

Experiment 5

The mean penetration of formalinized spermatozoa was 9 ± 6 (s.d.) μm . The maximum penetration measured was 18 μm .

Discussion

The results of this study are similar to those of Strzemiński *et al.* (1987) who studied horse spermatozoa; penetration into the filters increased with increased pore size and increased incubation time up to 60 min. On the basis of these findings the study was continued using 5 μm filters incubated for 60 min.

Strzemiński *et al.* (1987) demonstrated that formalinized stallion spermatozoa did not enter the filter; this was not confirmed by the present study with dog spermatozoa, even though the sperm dimensions are similar in these two species (Cummins & Woodall, 1985). The passage of dead spermatozoa into the filter produces a consistent minimum penetration distance although this should not influence the 'leading front' principle.

Dilutions of up to 1:4 produced an increase in the penetration of spermatozoa into the filter which probably reflects an increase in sperm velocity (Makler *et al.*, 1979). A 1:8 dilution produced penetrations similar to those of raw undiluted semen and since computer analysis of sperm velocity cannot be carried out using raw semen, a dilution of 1:8 was chosen. Greater dilutions produced a fall in sperm penetration into the filter which probably reflects the dilution effect (Emmens & Blackshaw, 1956; Mann, 1964). With long periods of storage of diluted samples it was possible to monitor the deterioration in sperm motility. The percentage of live and morphology normal spermatozoa was significantly reduced following 2 h of storage but there were still many live morphologically normal spermatozoa most of which were non-motile. This discrepancy between the vital staining and motility has been observed by other workers (Hong *et al.*, 1988). The accuracy of eosin/nigrosin staining in determining the proportion of dead spermatozoa in a sample has been demonstrated (Bangham & Hancock, 1955). These workers filtered samples of diluted bull spermatozoa and found that dead spermatozoa were retained by the filter; comparison of the pre- and post-filtering sperm counts correlated well with the supravital staining of the sample. The principle of supravital staining is that the stain is taken up by a cell with a damaged plasma membrane, whereas a functional membrane resists permeation. It is therefore likely that the non-motile spermatozoa in the present study have intact membranes.

The distance penetrated into the filters was significantly reduced after storage. However, the most sensitive indicator of deterioration of motility of the semen sample was the fall in mean sperm velocity. The fall in percentage motility was the next best indicator. These results demonstrate that cellulose acetate/nitrate filters can be used to quantify dog sperm motility, although the measurement of mean sperm velocity and the assessment of percentage motile spermatozoa appear to be more sensitive.

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