Evaluation of ram semen motility by a swim-up technique

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The turbidity formed as ram spermatozoa swam up into a clear medium was measured using a colorimeter and this was used as an objective index of sperm motility. Wavelengths of 470–475 nm yielded higher optical densities (OD) than did 520–640 nm. The recommended distance between the light beam and semen–medium interface, and operation temperature were 5 mm and 30–37°C, respectively. Of seven media tested, Tris–glucose was preferred on the basis of results, availability and cost. Diluting semen before testing reduced OD, whereas increasing semen concentration (0.6 to 2.4×10^9 spermatozoa ml^{-1}) and the total number of spermatozoa used increased OD. OD gradually increased with time in all experiments ($P < 0.001$). High positive correlations between OD and known percentages of motile spermatozoa (0.82 – 0.91 at from 5 to 20 min, all $P < 0.001$) suggest that this swim-up technique, which is objective and inexpensive, is valid for measurement of sperm motility in rams.

Introduction

Visual assessment of sperm motility is simple, rapid and inexpensive, but its subjective nature leads to variation between observers and over time. A number of objective methods of assessing motility have been developed (Rothschild, 1948, 1953a, b; Dott, 1975), but are time consuming and relatively expensive. Sokoloski *et al.* (1977) proposed changes in optical density (OD) as a means of estimating the velocity of spermatozoa swimming into a medium, and this basic approach led us to develop an alternative procedure which we refer to as a swim-up technique.

This paper reports on experiments designed to investigate factors that could be expected to affect the rate of OD increase as spermatozoa swim into a medium: wavelength, temperature, media, the presence of seminal plasma, semen dilution, semen concentration, and number of spermatozoa present. The potential of the method as an objective measure of semen motility was assessed by making observations on a series of semen samples containing known proportions of motile and immotile spermatozoa.

Materials and Methods

Semen

Semen from adult Merino rams with initial motility (Salamon, 1976) of at least 90% was normally diluted with 300 mmol Tris l^{-1} –28 mmol glucose l^{-1} (TG) to 2×10^9 spermatozoa ml^{-1} and held at 37°C. The percentage of progressively motile spermatozoa was estimated in a layer of diluted semen 60 μl thick at $\times 150$ and 37°C.

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Cuvettes and colorimeter

Semen was layered at the bottom of modified, disposable cuvettes (Suttiyotin and Thwaites, 1992) and OD readings were routinely made at 5 min, or as otherwise indicated; the cuvettes and colorimeter were maintained at 37°C. The absorption spectra of seminal plasma, Tris–glucose and semen diluted with TG was established by using an automatic spectrophotometer fitted with a sample sipper and data plotter.

Media

Seven well-known media were studied: (i) Krebs Ringer bicarbonate with 0.2% BSA (K1: prepared in the laboratory, 323 mosmol, pH 7.4; K2: Sigma Chemical Co., Sydney; 267 mosmol, pH 7.0), (ii) ova maintenance medium (OMM: CSL, Melbourne, 306 mosmol, pH 7.0), (iii) Ham's F-10 (HAM: ICN Flow, Sydney, 250 mosmol, pH 7.0); (iv) modified Tyrode's solution (MT: Stewart-Savage and Bavister, 1988; 315 mosmol, pH 7.0); (v) BWW (Biggers *et al.*, 1971; 308 mosmol, pH 7.0), (vi) Tris–glucose (TG, 328 mosmol, pH 7.0); and (vii) Tris–fructose (TF, 356 mosmol, pH 7.0: both after Salamon and Visser, 1972).

Wavelength

Latin-square designs were used to study OD changes in semen diluted 1:1 with K1 at 490, 520 and 540 nm. A flow through cuvette (Sokoloski *et al.*, 1977) was used and readings were made every 10 s for 5 min. Undiluted semen was also observed at 470, 490, 520 and 540 nm using TG medium. The absorption spectra of semen diluted with TG (25 , 75 and 150×10^6 spermatozoa ml^{-1}) and TG alone were established at from 190 to 900 nm.

The absorption spectra of seminal plasma was similarly examined, using two replicates, in each of which two ejaculates

from each of five rams were pooled and centrifuged at 2000 *g* for 15 min. The supernatant was recentrifuged until examination by microscope revealed that the seminal plasma contained no spermatozoa. Spermatozoa obtained from the sediment after centrifugation were then washed three times in TG before being resuspended in TG to yield a suspension of 25×10^6 spermatozoa ml^{-1} which was scanned for absorption as before.

Distance from semen–medium interface to light beam

OD was measured after adjusting the distance between the lower edge of the light beam and the semen–medium interface to 1, 3, 5, 7 or 9 mm by means of a vernier cuvette support attached to the colorimeter (Suttiyotin and Thwaites, 1992).

Temperatures

Semen from each of four rams was diluted and allowed to swim up into TG at either 5, 10, 20, 30 or 37°C and OD was recorded.

Media

Comparisons of the efficacies of K1, OMM, TG and TF (undiluted semen), of K2, OMM, TG and TF (undiluted semen), and of HAM, MT, BWW and TG (diluted semen) were made at 470 nm. The possible effects of glucose in the medium were investigated by allowing four replicate subsamples of undiluted semen from four ejaculates to swim up into Tris solutions (300 mmol l^{-1}) supplemented with 0, 0.05, 0.1, 0.5 and 1% glucose.

Seminal plasma

On three separate occasions, ejaculates from two rams were pooled and centrifuged at 600 *g* for 15 min before supernatant and spermatozoa were separated. The seminal plasma was prepared by recentrifuging the supernatant at 2000 *g* for a further 15 min and discarding the sediment. The sperm pellet was resuspended in TG to the original volume and divided into two aliquots, one of which served as a motile sperm suspension (control) while the other was shocked in an 80°C water bath for 15 min and served as an immotile (absence of progressive motility confirmed using a microscope) sperm suspension. OD was measured using seminal plasma and both the motile and immotile sperm suspensions.

Semen dilution

The semen from three rams was used either undiluted or diluted 1:1, 1:2 or 1:3 with TG. The number of spermatozoa in each treatment was standardized by using respective volumes of 0.05, 0.10, 0.15 and 0.20 ml. The possible effects of different concentrations of seminal plasma in diluted semen under study were examined in suspensions of 2×10^9 spermatozoa ml^{-1} in TG or seminal plasma alone, or with 10 or 20% seminal plasma in TG. Four replicate subsamples from each semen suspension were used in each of three replicates.

Semen concentration

Pooled ejaculates from two rams were diluted with TG to $2.4, 1.2, 0.8$ and 0.6×10^9 spermatozoa ml^{-1} and aliquots containing 120×10^6 spermatozoa (50, 100, 150 and 200 μl , respectively) were tested in each of four replicates. Perspex blocks of different thicknesses were placed under the cuvettes to standardize the distance between the semen–medium interface and the lower edge of the colorimeter light beam at 5 mm.

Total number of spermatozoa

Ejaculates from each of four rams were diluted with TG to 1×10^9 spermatozoa ml^{-1} and four replicated subsamples containing 50, 100, 150 or 200×10^6 spermatozoa were tested.

Correlation with known percentage of motile spermatozoa

Pooled semen from four rams was centrifuged, washed with TG and frozen–thawed a number of times until examination by microscope ($\times 150$) revealed no motility. The spermatozoa were then rewashed and suspended in TG at 2×10^9 spermatozoa ml^{-1} to form an ‘immotile’ cell suspension (Suttiyotin and Thwaites, 1991). A ‘motile’ cell suspension was formed by diluting fresh semen from four other rams to 2×10^9 spermatozoa ml^{-1} with TG and subjectively assessing the progressive motility (mean 97.5%). Four replicate test samples (200×10^6 spermatozoa) containing known percentages of ‘motile’ cells were prepared by mixing the ‘immotile’ and ‘motile’ cell suspensions in the ratios 1:0, 3:1, 1:1, 1:3 and 0:1.

Loss of motility during the swim-up procedure

The extent of loss of sperm motility was checked during the 20 min required for swim-up assessment by evaluating samples from two sources for progressive motility using a microscope. First, in several of the preceding experiments, progressive motility was checked before loading into the cuvette, and again after mixing the contents of the cuvettes after a 20 min test. Second, a dummy run of the swim-up procedure was conducted, using four pooled semen samples, three replicate cuvettes per time, and three observers. Duplicates of each semen sample, as loaded into the cuvettes, were held in three small test tubes at 37°C as controls. Subsamples were withdrawn from the bottom of the cuvettes and the corresponding test tubes at 0, 5, 10, 15 or 20 min after loading of the semen and the percentage of progressive motility was estimated by three observers at the same time using a projecting microscope ($\times 125$).

Statistical analysis

The data, after appropriate angular transformation, were analysed by a computer programmed analysis of variance for repeated measures. Where a significant difference between means was observed, it was tested by Duncan’s new multiple range test (Steel and Torrie, 1980) and linear regression analysis was used to relate the known percentage of motile spermatozoa in a test suspension to OD when applicable.

Table 1. Means of optical density readings of diluted semen (spermatozoa, seminal plasma and TG), seminal plasma alone and spermatozoa diluted in TG (no seminal plasma)

Wavelength (nm)	Diluted semen	Plasma	Spermatozoa + TG
640	1.70 ± 0.23 ^a	0.38 ± 0.10 ^a	1.37 ± 0.19 ^a
610	1.71 ± 0.23 ^a	0.43 ± 0.08 ^{ab}	1.39 ± 0.19 ^{ab}
578	1.72 ± 0.23 ^a	0.50 ± 0.09 ^b	1.41 ± 0.19 ^b
520	1.76 ± 0.23 ^b	0.67 ± 0.11 ^c	1.47 ± 0.19 ^c
475	1.84 ± 0.22 ^c	0.89 ± 0.14 ^d	1.54 ± 0.19 ^d
440	1.91 ± 0.22 ^d		
400		1.69 ± 0.19 ^e	1.73 ± 0.19 ^e

TG: 300 mmol Tris l⁻¹–28 mmol glucose l⁻¹.

Values are means ± SEM.

Means within columns with different superscripts differ significantly ($P < 0.05$).

Results

Wavelength

Differences in OD between wavelengths were not significant (0.07–0.08 in diluted semen at 490–540 nm and 0.49–0.50 in undiluted semen at 470–540 nm). Neither TG nor semen diluted with TG showed any distinctive absorption peaks within the visible range of 400 to 700 nm. For semen diluted with TG, OD increased from 1.70 at 640 nm to 1.91 at 440 nm (Table 1). Below 400 nm, the OD of both TG and semen diluted with TG increased dramatically to values > 3.0 . Seminal plasma and the suspension of spermatozoa in TG yielded similar spectra to diluted semen and in both cases OD increased as wavelength was reduced from 640 to 578 nm (Table 1).

Distance from semen–medium interface to light beam

Distances of 1 and 3 mm yielded OD values of 1.10–1.38 during the 20 min test. At 5 mm, OD increased progressively to reach a plateau value of 0.78 after 10 min, whereas at 7 and 9 mm the rate of OD increase was much less. After 5 min, OD at 5 mm (0.35 ± 0.13) was higher than at 7 and 9 mm (0.12 ± 0.07 and 0.17 ± 0.01 , respectively, $P < 0.05$).

Temperature

At 20–37°C, OD increased progressively with time, but at 5 and 10°C, values were constant and low (0.01–0.09). The overall OD at 37°C (0.78 ± 0.07) was lower than at 30°C (0.82 ± 0.07 , $P < 0.05$) but higher than at 20°C (0.30 ± 0.06 , $P < 0.001$).

Media

OD readings using OMM and TF were not significantly different (0.54 ± 0.05 and 0.60 ± 0.03 , respectively), but both were lower than in TG (0.78 ± 0.02 , $P < 0.001$ and $P < 0.01$, respectively) and higher than in K1 (0.10 ± 0.01 , $P < 0.001$).

Fig. 1. Optical density of ram spermatozoa during swim up in Tris medium containing (□) 0, (▨) 0.05, (▩) 0.10, (▧) 0.50 and (■) 1.00% glucose. **Fig. 2.** Optical density of (□) seminal plasma and (▨) motile and (■) immotile cell suspensions in the swim-up technique.

OD readings using K2 and OMM did not differ significantly (0.39 ± 0.04 and 0.43 ± 0.05 , respectively), but those for K2 were lower than for both TF (0.41 ± 0.02 , $P < 0.005$) and TG (0.47 ± 0.03 , $P < 0.001$). BWB yielded lower OD (0.19 ± 0.02) than MT, HAM and TG (0.22 ± 0.02 , 0.26 ± 0.02 and 0.28 ± 0.02 , respectively, $P < 0.05$).

The presence of glucose in Tris medium produced greater OD responses than Tris medium alone (Fig. 1, $P < 0.001$). OD progressively increased with increasing glucose concentrations ($P < 0.005$).

Seminal plasma

Seminal plasma and the immotile sperm suspension produced similar OD values (Fig. 2), both of which were lower than for the motile spermatozoa suspension ($P < 0.001$).

Semen dilution

The mean OD values recorded when ram semen was diluted 1:1, 1:2 or 1:3 with TG (0.33 ± 0.02 , 0.32 ± 0.02 and

Fig. 3. Relationships between known proportions of motile and immotile spermatozoa and optical density readings at (a) 5 min, (b) 10 min, (c) 15 min and (d) 20 min.

0.32 ± 0.02 , respectively, n.s.) were less than for undiluted semen (0.49 ± 0.03 , $P < 0.001$). Increasing the seminal plasma concentration increased OD: although the mean OD of samples containing 0 and 10% plasma did not differ (0.38 ± 0.02 and 0.40 ± 0.02 , respectively), those for 20 and 100% plasma were increased (0.46 ± 0.03 and 0.54 ± 0.02 , respectively, $P < 0.001$).

Semen concentration

OD gradually increased with increasing semen concentration; although OD at 0.6 and 0.8×10^9 spermatozoa ml^{-1} did not differ (0.40 ± 0.03 and 0.41 ± 0.03 , respectively), both values were lower than that at 1.2 (0.47 ± 0.03 , $P < 0.05$) and 2.4×10^9 spermatozoa ml^{-1} (0.59 ± 0.03 , $P < 0.001$).

Total number of spermatozoa

The number of spermatozoa deposited had a positive effect on OD; mean values were 0.21 ± 0.01 , 0.37 ± 0.03 , 0.57 ± 0.04 and 0.74 ± 0.04 for 50, 100, 150 and 200×10^9 spermatozoa, respectively (all $P < 0.001$).

Correlation with known percentage of motile spermatozoa

Regression analyses revealed that the correlations between the proportion of motile spermatozoa and OD at each time point were homogeneous and the data were thus pooled for each time and re-analysed. The resultant correlations between

the percentage of motile spermatozoa and OD at 5, 10, 15 and 20 min were 0.82, 0.86, 0.87 and 0.91, respectively (all $P < 0.001$). The relationships between these variables are presented (Fig. 3) and the application of Bartlett's test (Snedecor and Cochran, 1967) revealed that the variances within times were not significant.

Loss of motility

Motility loss during 20 min of the swim-up procedure in the preceding experiments ranged from 9.7 to 20.0% except for K1 (59%). In the dummy run of the technique, the percentages of motility lost at 5, 10, 15 and 20 min were not significantly different (8.2 ± 2.7 , 6.2 ± 1.8 , 4.9 ± 2.0 and 4.0 ± 1.6 , respectively). There were no significant differences between either ejaculates or observers. However, the percentage of motility lost was lower in the test tubes (1.2 ± 1.4) than in the cuvettes (10.4 ± 1.4 , $P < 0.001$).

In all experiments OD increased with time ($P < 0.001$).

Discussion

The ability of spermatozoa to swim up into a medium has been used extensively in the selection of motile cells (Lopata *et al.*, 1976; Berger *et al.*, 1985; Tanphaichitr *et al.*, 1987) for *in vitro* fertilization. The same principle was later used to evaluate sperm motility (Sokoloski *et al.*, 1977; Levin *et al.*, 1980, 1981,

1984) by measuring turbidity of the medium as spermatozoa swam into it.

The wavelengths used by earlier workers ranged from 475 (Atherton *et al.*, 1978) to 545 (Sokoloski *et al.*, 1977; Majumder and Chakrabarti, 1984; Sidhu and Guraya, 1990), 550 (Jasko *et al.*, 1989) and 580 nm (Deana *et al.*, 1986). As a general rule, accuracy and sensitivity will be greatest if the wavelength used yields maximum OD and corresponds to any specific absorption peaks (Skoog, 1985). Optimum wavelengths were determined by scanning samples across an appropriate range. In the swim-up technique, OD necessarily changes with time and normal scanning is thus not possible. Three alternative approaches were used in the work reported here. First, measurements at fixed times failed to detect any difference in OD with the four colorimeter filters used, which had, however, a bandpass of 40 nm which may have masked small variations in OD. However, no specific absorption peaks in the visible light range were seen in the spectrophotometer scans. In contrast, strong absorption peaks (OD > 3.0) were observed in the ultraviolet (UV) range (190–400 nm).

Atherton *et al.* (1978) scanned human seminal plasma and reported significant interference from seminal plasma and debris in the UV range. To minimize such effects, they suggested the use of a wavelength of 475 nm. Similar effects of seminal plasma were observed in the study reported here; interference was lower at 640 nm than at 610, 578, 520 or 475 nm. The absorption spectrum of ram seminal plasma observed in the present study was similar to that reported by Dott (1974) for the bull. Although high wavelengths minimize interference from seminal plasma, they concurrently reduce OD readings and thus lower accuracy. In contrast, low wavelengths yield higher OD readings, but are subject to interference. However, the current work indicates that neither seminal plasma nor immotile spermatozoa increased OD in the swim-up technique and the use of 475 nm is recommended. At 475 nm high OD readings are obtained and this wavelength corresponds to that used by Atherton *et al.* (1978) for human semen and to the 'blue' filters recommended for colorimetric evaluation of ram semen by Emik and Sidwell (1947) and Salamon (1976). The increase in OD with increasing wavelength when scanning a suspension of spermatozoa in TG contrasts with a plateau spectra for TG alone and indicates that the increase in OD results from spermatozoa themselves.

The high initial OD readings when the distances of 1 and 3 mm were used between the lower edge of the light beam and semen–medium interface suggest that these distances were too small to be practical. Each of the distances 5, 7 and 9 mm produced increasing OD over time, but 5 mm yielded the highest reading at 5 min and is thus preferred.

Temperatures of 5 and 10°C yielded only low OD values and are not suitable for this technique. However, temperatures of 30–37°C are recommended for use since they yielded higher OD than 20°C and correspond to those normally used in semen processing.

K1 was inferior to OMM, TF and TG, possibly because it had a lower concentration of glucose. Although significant differences were observed in the efficacy of the media, mean differences in OD at 5 min (except for K1) were small (0.08 ± 0.03) and would not be expected to influence readings in the swim-up technique significantly. Apart from K1, the media were thus

suitable for use. For ease of use, particularly in the field, Tris is both simple to prepare and store and supports sperm survival better than other buffered media studied (Davis *et al.*, 1963; Gebauer *et al.*, 1970) and glucose is cheap and widely available. As spermatozoa also tolerate a relatively wide range in concentration of Tris solution (Steinbach and Foote, 1967; Yassen and Foote, 1967; Igboeli, 1970; Salamon and Visser, 1972), TG was selected for routine use in the swim-up technique.

Neither immotile spermatozoa nor seminal plasma alone caused significant changes in OD (Fig. 2) and we thus conclude that the observed increases in OD in the swim-up technique are due to the upward movement of motile spermatozoa. This is consistent with the results of Sokoloski *et al.* (1977), who concluded that the settling speed of nonmotile cells was negligible compared with the swimming speed of motile cells.

A number of workers have reported that the dilution of semen reduces sperm motility (Cheng *et al.*, 1949; Bavister, 1974) and fertility (Lapwood *et al.*, 1972; Martin and Watson, 1973, 1976; Watson and Martin, 1976), although others have failed to distinguish any such effects (Jones *et al.*, 1969; Entwistle and Martin, 1972). In the current work, a negative effect of dilution (1:1 to 1:3) on OD was clearly indicated, although the design initially used confounded the effects of dilution and semen concentration. This problem was subsequently rectified by standardizing the semen concentration; the results were consistent with earlier ones and indicated that 20% or more seminal plasma in the semen sample was beneficial in terms of the OD recorded. Seminal plasma is known to stimulate sperm motility (Lindholmer, 1974; Bavister and Yanagimachi, 1977; Bavister *et al.*, 1978; Dott *et al.*, 1979; Tso and Lee, 1980; Baas *et al.*, 1983) and the current results indicate that using it as a diluent would increase OD values. However, preparation of seminal plasma is slow and costly, and its use would, in addition, possibly mask any deficiencies in the seminal plasma of the semen sample being tested. On balance, its use as the diluent in the swim-up technique is thus contra-indicated.

Our results indicate that semen concentration (number of spermatozoa available at the semen–medium interface) positively influenced OD. This is the first report of such an effect, and it supports the suggestion that the greater the number of spermatozoa available to swim up, the quicker the rise in OD and the higher the eventual values achieved. Although a high semen concentration would thus be desirable, compromise is necessary to take into account the range of semen concentrations encountered in the field. Most authorities (e.g. Salamon, 1976; Setchell, 1991) agree that 2×10^9 spermatozoa ml^{-1} represents the lower limit of normal semen concentration in rams. This value is thus recommended for routine use in the swim-up technique for rams; in most cases it will involve dilution only at a rate of between 1:1 and 1:2.

The number of spermatozoa available had a positive and marked effect on OD. After 20 min, for example, OD for the 200×10^6 spermatozoa loading (0.98 ± 0.04) was more than three times higher than for 50×10^6 spermatozoa (0.27 ± 0.02). Such a response is in agreement with results of Deana *et al.* (1986), who found that the OD after 20 min varied from 0.4 to 0.9 with loadings of 0.05 and 0.15 ml of bull semen (concentration not specified). Majumder and Chakrabarti (1984) reported a similar response in goat semen, although their semen loadings and OD values were much lower than those used in

this study. Since the current work began, Jasko *et al.* (1989) have also reported a positive effect of sperm number on OD, although differences in technique make comparisons difficult. For routine use in the swim-up evaluation of ram semen, a sperm loading of 200×10^6 cells is recommended. The fact that variances within times did not differ significantly indicates that each of the times (5–20 min) studied yielded a similar level of accuracy. On the basis of the speed of operation and ease of reading the colorimeter, 5 min is recommended for the duration of the proposed swim-up technique. In addition, the negligible loss of motility for up to 20 min of the technique indicates that the conditions used (TG as a medium, 37°C) were quite adequate to support spermatozoa. Had conditions been completely optimal, no differences in motility would have been expected between cuvettes and test tubes. In fact, semen in the cuvettes suffered, on average, a 9.2% greater motility loss than control samples in test tubes ($P < 0.001$). Loss of motile spermatozoa from the semen sample into the TG medium does not appear to have been a major contributor to the slight loss in motility observed, since differences with time were not significant.

Owing to failure to standardize the conditions under which swim-up type evaluations of sperm motility have been made in the past (Sokoloski *et al.*, 1977; Majumder and Chakrabarti, 1984; Deana *et al.*, 1986; Jasko *et al.*, 1989; Sidhu and Guraya, 1990), it has not previously been possible to compare the results from different laboratories. The work reported here clearly demonstrates the need to standardize the wavelength and media used, the concentration and volume of semen loaded into the cuvette, the time allowed for each test, and the distance between the semen–medium interface and the light path through the colorimeter cuvette. In a number of colorimeters examined, the last distance varied from 8 to 15 mm. For the swim-up technique, a standard distance of 5 mm is thus recommended, as it will accommodate most instruments, and can readily be achieved by placing an appropriate spacer block at the bottom of the cuvette well.

The following standard procedure for the swim-up technique is recommended: 0.1 ml of semen previously diluted with TG to 2×10^9 spermatozoa ml^{-1} is layered at the bottom of a cuvette modified by the inclusion of an injection port and which contains 2 ml TG. OD is then read at 470–475 nm after 5 min; the distance between semen–medium interface and colorimeter light path is adjusted to 5 mm, and the temperature standardized throughout at 30–37°C.

This swim-up technique was shown to be valid in objectively evaluating semen motility as it was highly correlated with the known proportions of motile and immotile spermatozoa in the test samples, and with parameters from a Hamilton Thorn Motility Analyser (Suttiyotin *et al.*, 1991; Suttiyotin and Thwaites, 1992).

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