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Assessment of spermatozoal function

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Abstract. Different procedures have been developed to assess the functions of spermatozoa in terms of their motility as well as their fertilizing potential. The procedures for assessment of motility are either qualitative or quantitative, subjective or objective, while the procedures for assessment of fertilizing potential are either direct or indirect. In this review, the information available on the procedures are compiled and analysed including the procedures for *in vitro* penetration of zona-free hamster eggs by human or non-human primate spermatozoa.

Keywords. Sperm motility; objective assessment; fertilizing ability; hybrid fertilization; indirect assessment.

Introduction

Assessment of sperm function is essential for research into new methods of fertility regulation in male and for the investigation and management of infertile male. The acceptance and dissemination of the results of scientific advnaces depend upon the replication and confirmation of results by investigators in different institutions and therefore standardisation of procedures for assessment of sperm function is necessary.

Mammalian spermatozoa released in the lumen of the seminiferous tubules appear highly differentiated but continue to develop during their passage through the epididymis and acquire a mature motility pattern and fertilizing ability. Whether any other molecular or functional changes occur during storage or when mixed with the secretions of the accessory genital organs at the time of ejaculation remain to be properly elucidated. However, the spermatozoa undergo other important transformations in the female genital tract including capacitation, acrosome reaction, species-specific binding and penetration of the zona pellucida before fusing with the egg at the time of fertilization. The differentiations and transformations of the spermatozoa are ultimately aimed at fertilization of eggs. The development of the mature motility pattern endows the spermatozoa with the capacity to negotiate the passages in the male and female genital tract and probably the modified motility pattern of capacitated and acrosome reacted spermatozoa is required for attachment and penetration of the zona pellucida.

The functional status of spermatozoa is usually assessed when they are obtained

Abbreviations used: PR, Progressiveness ratio; SLD, straightline distance; TD, total distance.

from the terminal segment of the epididymis of laboratory animals where the matured spermatozoa are stored or from the semen of primates including human at ejaculation. The parameters of assessment are concatanated either with motility or with fertilizing ability of spermatozoa. However, the concentration and morphology of spermatozoa are also important as these have correlation with fertility of the individual.

Assessment of sperm motility

Motility of epididymal spermatozoa

The acquisition and change in the pattern of motility of spermatozoa during epididymal transit have been amply documented for many years (Tournade, 1913; Blandau and Rumery, 1964; Gaddum, 1968).

The spermatozoa are usually collected by isolating the epididymis, making multiple punctures, taking out the exudates and examining the motility under the light microscope. The motility is observed either suspended in the exudate or after dilution with medium which may be either physiological saline or any other nutrient medium. The pattern of motility may be classified as vibratory, circular, darting forward and rotatory (Gaddum, 1968; Fray *et al.,* 1972; Das *et al.,* 1973). Without exceptions, all studies of mammalian sperm movement to date have involved observations *in vitro.* They are observed on microscope slides with or without cover glasses, capillary tubes of circular or rectangular cross-sections or other similar chambers. There have been many attempts to speed up the visual measurements of sperm motility and make it objective. Spectrophotometry (Timourian and Watchmaker, 1970), time-lapse photometry and cinematographic recording (Amann and Hammerstedt, 1980; Overstreet *et al.,* 1979), scattered laser light (Finsy *et al.,* 1979), scanning by television camera (Suarez and Katz, 1982), scanning with an image-analyzing computer-linked microscope (Katz and Dott, 1975), have been reported to reduce subjectivity in evaluation of sperm motility and some of these may be discussed in details in subsequent section. Visual assessment, however, continues to be the dominant method used to judge sperm movement and scores are assigned to samples on a purely arbitrary scale.

Motility in the epididymal fluid: The relationship between the movements that spermatozoa are capable of exhibiting *in vitro* and their actual behaviour *in vivo* remains to be determined. Sperm movement characteristics may alter depending on the chemical and physical properties of the suspending media, as well as to the geometrical, material and electrochemical characteristics of the chambers within which they reside (Katz *et al.,* 1978; Lindemann, 1978). Large species differences are apparent if the spermatozoa are taken and incubated in their epididymal fluid (table 1).

The number of motile spermatozoa is zero in the boar or very low at all epididymal levels in the rat or mouse (Wyker and Howards, 1977; Morton *et al.,* 1978; Turner *et al.,* 1978). However, in human and rabbits (Morton *et al.,* 1978) or rams (Dacheux and Paquignon, 1980) motile spermatozoa appear in the corpus epididymis and their number and motility increase in the cauda epididymis.

Potential motility: In some species (mouse, rat) the immobile spermatozoa in the epididymal fluid develop motility after dilution (Morton *et al.,* 1978; Turner *et al.,*

Table 1. Motility of spermatozoa incubated in their own epididymal fluid (Dacheux and Paquignon, 1980).

Species	Sperm origin				References
	Testis	Caput ep.	Corpus ep.	Cauda ep.	
Boar	—	—	—	—	Dacheux and Paquignon, 1980
Rat	—	—	—	(+)	Morton *et al.*, 1978
Mouse	—	—	—	(+)	Morton *et al.*, 1978.
Bull	—	—	+	++	Morton *et al.*, 1978.
Ram	—	—	+	+++	Dacheux and Paquignon 1980.
Rabbits	—	(+)	+	++	Morton *et al.*, 1978.
Man		+	++	+++	Morton *et al.*, 1978.

1978). The number of motile spermatozoa increases regularly between the caput and the cauda epididymis.

Motility of ejaculated spermatozoa

The studies on motility of ejaculated spermatozoa are mostly restricted to domestic animals and primates including human. The semen samples may be obtained either by electro-stimulation with electrical impulses (electraejaculation) or by teasing with artificial vagina or by masturbation (in human).

The collection of semen samples require certain precautionary measures. According to the Manual of WHO (1980) the following precautions are necessary.

(i) The sample should be collected after a two to seven day period of abstinence.
(ii) Two to three specimens should be collected at weekly or biweekly intervals.
(iii) The entire sample should be collected at the laboratory.
(iv) The entire sample should be obtained by masturbation directly into a clean glass or plastic container which has been prewarmed to room temperature before use.
(v) Rubber stoppers or rubber condoms must not be used.
(vi) Coitus interruptus is not acceptable as a means of collection.
(viii) The sample should be protected from extremes of temperature (not less than 20° C and not more than 40°C) from the time of collection to the time of examination in the laboratory.

The period of abstinence may not have any relation with motility in human (Mortimer *et al.*, 1982). However, the mean ejaculate volume increases by 1·0 ml between the first and second days of abstinence, and at approximately 0·3 ml/day thereafter until the fifth day. The total sperm count increased by 50–60 × 10^6 spermatozoa/day during this period.

Visual estimation of motility (WHO Manual, 1980): Before the microscopic examination is performed, it is absolutely necessary to have a well-mixed specimen. The mixing is not easily accomplished by using a vortex mixer. One drop, approximately 2–3 mm in diameter, is placed on a clean glass slide, covered with a small cover slip and examined under a magnification of 400–600 X in a light microscope, preferably under place

contrast. The slide must be examined immediately in a chamber with an ambient temperature of 37°C or on a temperature controlled microscope plate having temperature of 37°C.

Quantitative motility is determined by counting both motile and immotile spermatozoa in at least 10 separate and randomly selected fields (but not near the edge of the cover slip). At least 100 spermatozoa must be counted. The percentage or motile spermatozoa is calculated from the mean percentage motility for all fields counted and adjusted to nearest 5 %.

Qualitative motility is determined subjectively by grading the forward progression made by the largest numbers of spermatozoa. The gradations are 'none, poor, good and excellent.' A normal sample will show 60 % or greater motile spermatozoa with the majority exhibiting good to excellent forward progressive movement at 30 min to 3 h after ejaculation.

Objective assessment of sperm movement characteristics

Assessment of speed of movement

The simplest objective assessment of sperm movement characteristics is the measurement of the speed (Daunter *et al.,* 1981). Spermatozoa suspensions are checked at 5, 10 or 15 min intervals under a light microscope having an eye-piece graticule with cross-lines at 90° at 400 X magnification. The distance from the centre of the cross-lines of the eye-piece graticule to the periphery is measured using a stage micrometer. The time taken by a spermatozoon to move in a straight line from the centre of the cross-line of the eye-piece graticule to the periphery of the field of view is recorded using a stopwatch. The procedure is repeated for 10 spermatozoa in a given field of vision in each of the samples and the average forward velocity (μm/sec) is computed and the results expressed as mean ± S.E.

Spectrophotometric analysis of sperm motility

The method indirectly measures the degree of motility of spermatozoa. Sperm suspensions are diluted in physiological medium in cuvettes of spectrophotometer and at different time intervals the turbidity due to dispersal of spermatozoa is measured in a spectrophotometer at 560 nm at different set intervals. The degree of turbidity is plotted against time to give curve (Timourian and Watchmaker, 1970; Atherton *et al.,* 1978; Levin *et al.,* 1984).

Time-exposure photography for assessment of sperm motility

A simple, objective method for obtaining a well-defined sperm movement characteristics is described by Overstreet *et al.* (1979). One second time exposure photomicrographs are taken with dark-field illumination of slides prepared from semen, cervical mucus or artificial media. The negatives are projected as a filmstrip and are analyzed on a specially designed console. Developed filmstrips are projected at a final magnification of 1,000 X and trajectories plotted on paper. Analysis of these tracks give the percentages of immotile, nonprogressive, and progressively motile spermatozoa, along with the parameters *Vp* and *Ah* for the progressive spermatozoa (figure 1).

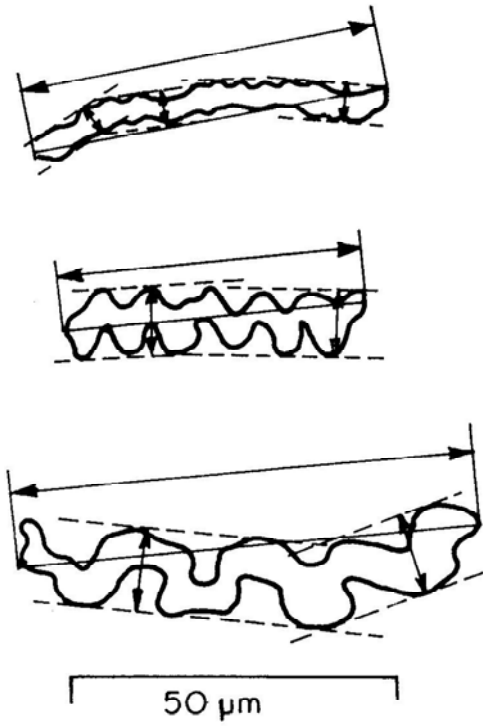

Figure 1. Diagrammatic representation of the method of analysis of tracings from 1 sec timed exposure filmstrips. Open arrows indicate how Vp (μm/sec) is obtained and solid arrows the measurements whose average for each spermatozoan is its Ah (μm).

When the visual assessment of percentage motility is compared with the percentage motility read from the photomicrographic negatives, the agreement is excellent.

The straightness of the swimming trajectory for an individual spermatozoon may be assessed directly from the tract or retrospectively from the progressiveness ratio (PR), *i.e.* the ratio between straight-line distance (SLD) and total distance (TD) of a track. Spermatozoa swimming along straight trajectories have a PR of 1. Spermatozoa with a PR of less than 1 may be swimming in a cricle and/or their progression may be erratic. Spermatozoa with circular motility show minimal or no evidence of yawning, whereas 'erratic' motility is invariably associated with a yaw component.

This technique can be utilized for study of human and monkey spermatozoa in semen and cervical mucus. No dilution of sperm suspension is necessary. The procedure measures per cent of motile sperm, swimming speed and per cent of spermatozoa having straight, rolling or yawning motility.

Microcinematographic measurement of sperm motility

According to this technique sperm movements are visualized using dry dark-field optics at a total magnification of 63 X. (Makler, 1978a, b, 1980; Katz *et al.,* 1978; Katz and Yanagimachi, 1980). The movement characteristics are assayed using high-speed

cinematography. Films are taken at 50 frames/sec using a 16 mm movie camera and film of 400 ASA. The processed films are analyzed frame by frame. Films also are taken at 10 frames/sec and trajectories are analysed (Makler, 1978b).

According to Katz and Yanagimachi (1980) films are taken at 50 frames/sec, beat shapes are traced and the beat frequency counted over the same interval. Two measures of sperm swimming speed are computed. The 'Straight line' velocity (VSL), is defined as the net distance swum per unit time along the straight line connecting the initial and final points on the trajectory. The 'curvilinear' velocity (Vc), is defined as the total distance per unit time traversed along the local position of the trajectory. The straightness of the trajectory is quantitatively measured by defining a "progressiveness ratio", PR = VSL/Vc (figure 2).

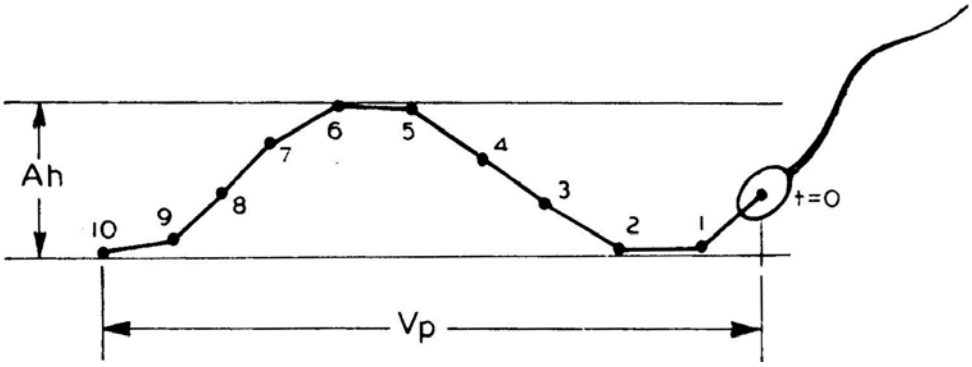

Figure 2. Diagrammatic representation of the method of analysis of 10 frames/s cine films of progressively motile human spermatozoa showing how the parameters Vp (μm/s) and Ah (μm) are measured (Makler, 1978b).

Laser Doppler velocimetry for study of sperm motility

The percentage of motile cells and their model instantaneous velocity in semen samples may be measured by Laser Doppler velocimetry (Dubois *et al.,* 1975; Finsy *et al.,* 1979). The measurement is made at 37°C using 40 μl volume in a glass cell of 200 μm depth. The instantaneous velocity of any moving spermatozoon is the real three- dimensional velocity of the head.

Mortimer *et al.* (1984) used 10 frames/sec microcinematography ('film'), 1 sec timed exposure photomicrography ('photo') and laser Doppler velocimetry (LDV) to analyse the swimming patterns of human spermatozoa after migration (1 h at 37°C) into an overlying layer of either BWW or Menezo's B2 media. Film and photo analysis give the relative incidence of non-progressive and progressively motile spermatozoa plus, for the progressive spermatozoa, the velocities of progression (VP) and amplitudes of lateral head displacement (Ah). LDV gave the percentage of motile spermatozoa and the model instantaneous velocity. Apparent discrepencies between the three methods used were seen.

A muti-channel optical system for objective evaluation of sperm motility is described by Mayevsky *et al.* (1980). The principle of the technique is based on the measurement of changes in the intensity of light scattered from semen samples thermo-regulated at 38°C. These changes are recorded continuously as analog waves (Reflecto-spermiogram-RSG). Changes in the intensity of sperm motility as a function of time from ejaculation are simultaneously measured in 8 equal semen samples from single ejaculate. The exposure of sperm cells to 366 nm light used in the system does not alter their ultrastructure as observed by electron microscopy. This objective, relatively easy to perform technique may be of value for research studies.

Assessment of sperm fertilizing ability

Fertility test

In laboratory animals the fertilizing ability of spermatozoa may be assessed by mating of males with females of proven fertlity and observing the outcome of pregnancy. The procedure requires observation till the end of the gestation period or otherwise the females may be laparotomized on day 10 of pregnancy and the number of implantation sites may be counted. This test cannot generally, be applied to primates and obviously to human because of many practical difficulties. In addition, this test is not specific for evaluation of fertilizing ability of spermatozoa since other factors in the semen and the female genital tract may contribute to the fertility of the animals.

In vivo fertilization test

The *in vivo* fertilization test can be carried out with spermatozoa of laboratory animals obtained from any part of the male genital tract and the test is more specific for determination of the fertilizing ability of spermatozoa. In short, spermatozoa are collected usually from the cauda epididymis. The sperm suspension after dilution with Tyrode's solution is injected into the uterus of females in heat and the eggs are recovered next morning and examined for the presence of two polar bodies, two pronuclei and sperm tail as indicators of fertilization (Das and Yanagimachi, 1978). Alternatively, the eggs can be flushed out of the oviduct 48 h after insemination and observed for 2 cell or 4 cell fertilized eggs (Warikoo and Das, 1983).

In vitro fertilization test

Since the recognition of 'capacitation of sperm' in the female tract (Austin, 1951; Chang, 1951), successful *in vitro* fertilization of mammalian eggs has been achieved in the rabbit (Thibault *et al.,* 1954; Chang, 1959; Brackett and Williams, 1968), hamster (Yanagimachi and Chang, 1963), mouse (Whittingham, 1968; Iwamatsu and Chang, 1969), rats (Miyamoto and Chang, 1973), guinea pig (Yanagimachi, 1972) and man (Edwards *et al.,* 1969, 1970). The various conditions for the fertilization of rat eggs *in vitro* is described by Niwa and Chang (1974) and that of hamster eggs by Leibfried and Bavister (1981). In rat, the spermatozoa need capacitation in female tract before they are capable of penetrating the egg. The time required for capacitation of rat sperm in the female tract appeared to be about 4–5 h. In hamster, capacitation of sperm may be

obtained in artifical media, the time required is 2 to 4 h. Normal fertilization is defined as presence of a single sperm in vitellus, with two pronuclei and two polar bodies.

The *in vitro* fertilization test for assessment of the fertilizing ability of spermatozoa has utility in terms of quick evaluation of the effect of any treatment of the males with potential antifertility agents. In addition, initial screening of large number of compounds may be carried out with spermatozoa from different animals treated with different agents and ova obtained by superovulation.

Zona -free hamster egg penetration tests

There are obvious logistical and ethical difficulties encountered for assessment of the fertilizing ability of human and monkey spermatozoa by fertility test. The *in vitro* assessment of the fertilizing ability of human and monkey spermatozoa should be ideally carried out using human and monkey eggs so that the ability of spermatozoa to penetrate the vestments of the oocyte and get incorporated in the oocyte can be determined simultaneously. However, there are technical and ethical problems related to the recovery of eggs and fertilization by spermatozoa of human origin (Prasad, 1984).

The hamster egg assay has received widespread attention in the evaluation of sperm fertilizing potential, especially in the human. When stripped of the surrounding cumulus cells and zona pellucida, this egg shows little, if any, specificity and fuse with appropriately preconditioned sperm from many mammals (Yanagimachi *et al.,* 1976). While the significance of cross-species gamete fusion is unknown, this bioassay at least measures the ability of sperm to undergo capacitation, the acrosome reaction, and fusion with the vitellus (Yanagimachi *et al.,* 1976; Talbot and Chacon, 1982). The zona-free hamster eggs are readily penetrated by heterologous spermatozoa but not zona-free rat (Quinn, 1979) or rabbit ova (Aribarg and Bedford, 1977).

The fertilizing ability of spermatozoa from different regions of the epididymis of the marmoset monkey was assessed by determining their rate of fusion with zona-free hamster ova *in vitro* (Moore, 1981).

The details of the procedure for ejaculated human spermatozoa has been reviewed by Prasad (1984). Semen samples are obtained generally by masturbation and used immediately on liquefaction for the zona-free hamster egg penetration assay. The spermatozoa are separated from the seminal plasma by either repeated centrifugation and resuspension or by a swim-up procedure. The second procedure may be superior to the centrifugation technique (Wolf and Sokolski, 1982).

Spermatozoa are generally capacitated at 37°C in an atmosphere of 5 % CO_2 in air at a concentration of 10–35 × 10^6/ml and the time required varies widely from 1 h (Barros *et al.,* 1979) to 18–20 h (Rogers *et al.,* 1979; Cohen *et al.,* 1982) according to the concentration of human serum albumin used.

The ova are collected from the oviducts of superovulated adult hamsters. In general about 20 ova are added to 100–300 μl droplets of the capacitated sperm suspensions under paraffin oil and incubated for 2–5 h at 37°C under an atmosphere of 5 % CO_2 in air. At the end of the incubation period, the ova are washed in BWW medium, transferred to a clean microscope slide and compressed under a cover slip mounted on 4 paraffin wax supports. The observation is made under phase contrast microscope and

the presence of swollen sperm heads with an attached or closely associated tail within the vitellus is considered to be the criterion of penetration. Usually 30–60 ova are scored per sample of semen.

Indirect assessment of fertilizing ability

Acridine orange fluorescence: A new and practical test for evaluating the fertility of a male subject has been developed (Tejada *et al.,* 1984). Semen smears stained with acridine orange are read on a fluorescence microscope. Sperm heads appear either green (fertile) or red (infertile). The per cent green correlates with neither actual sperm count nor motility, indicating that this test measures a new parameter of male fertility.

Semen adenosine triphosphate: The concentration of adenosine triphosphate per ml of ejaculate is significantly correlated with sperm concentration, number of motile spermatozoa per ml, capacity of spermatozoa to migrate against gravity, and *in vitro* potential for penetration of zona-free hamster ova (Comhaire *et al.,* 1983). These qualities suggest that measurement of semen ATP may be a possible biochemical method for the quantitation of actual fertilizing potential of the ejaculate.

Conclusions

Traditional methods of semen analysis include determination of sperm number, morphology and motility, the later being more or less subjective assessment. Such measurements, provide information on the normality of spermatogenesis but otherwise have poor predictive qualities in terms of the fertility status of the individual. In laboratory animals it is possible to study spermatogenesis directly by assessing the histology of testis while in monkeys and human the study of testicular biopsy may not be considered as a regular procedure. The fertilizing ability of the spermatozoa of laboratory animals may be assessed by fertility tests or by *in vivo* and *in vitro* fertilization test but an objective evaluation of the motility of spermatozoa is not being followed. A standardized procedure for such objective evaluation of sperm motility is essential, specially for monkey and human, for acceptance and dissemination of the results of scientific advances. A number of new and improved techniques have been developed but a consensus of opinion is necessary to decide about the most convenient and standard procedure to be followed. The standard procedure may as well be the principles laid down by Overstreet *et al.* (1979) and the characteristics of sperm movement measured may include determination of per cent motility, sperm velocity, the linearity of sperm progression and the amptitude of lateral head displacement. The number of spermatozoa possessing a certain characteristic of movement may be expressed as the percentage of the measured population or as an absolute concentration in the incubation mixtures (Aitken *et al.,* 1983).

The *in vitro* penetration assay utilizing human sperm and zona-free hamster eggs has been employed by a number of investigators (Prasad, 1984) but positive correlation is not obtained with the fertility status of the individual (Zausner-Guelman *et al.,* 1981; Cohen *et al.,* 1982 a,b). However, alongwith other indices of sperm function including motility, it may prove to be an important assay procedure for determining the fertility

status of an individual. The indirect assessment of the fertilizing ability of spermatozoa *e.g.* the ATP concentration in semen and acridine orange fluorescence may require confirmation by further studies with large sample size.

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