Gametes – The Spermatozoon

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Tests of sperm function

J. M. CUMMINS

Introduction

The past decade has seen rapid changes in the treatment and understanding of male infertility. Assisted conception techniques such *in vitro* fertilization (IVF), aspiration of spermatozoa from blocked epididymides and micromanipulation of sperm and ova give men with semen of very poor quality, or even total azoospermia, the opportunity to procreate. This is so even when we do not understand the underlying cause of the problem. These techniques are expensive, invasive and sometimes hazardous to the female partner, therefore we need to establish more accurate tests of the fertilizing potential of semen and to evaluate those tests in a critical and constructive manner. In this review I will argue that we must base effective functional tests of spermatozoa *in vitro* firmly on an understanding of the challenges faced by the sperm cell *in vivo*.

How do we define infertility?

One central problem in assessing any test of sperm function is that it is extremely difficult to assign an exact fertility status to any individual man. Is a man fertile if, at some stage in his life, he has initiated a pregnancy? This is not very realistic, as we know that fertility declines with age. In the classic series of studies that established 'normal' limits for semen, many of which are unchanged today Macleod, in his classic series of studies in the 1950s, defined men as fertile if their partners were pregnant at the time of testing. However, this definition did not take account of pregnancy outcome, and the paternal contribution to early pregnancy failure. The most rigorous definition of male *in*fertility is probably the demonstration of repeated fertilization failure in IVF, with no defects in the oocytes of the partner. To be absolutely sure that the oocytes are indeed normal, one must simultaneously demonstrate fertilization and normal embryogenesis

with fertile donor spermatozoa at the same time, but this can pose ethical and legal problems. Without such unequivocal evidence the best assessments of fertility or infertility in the general population will come from prospective long-term follow-up studies. As some men can achieve pregnancy through assisted reproduction but not through natural conception the term 'subfertile' is used for this group.

How do we assess tests of sperm fertilizing ability?

Boyers, Davis & Katz (1989) have stated, concerning tests of sperm function and their relation to fertility, 'Criteria that predict success *in vitro* should be regarded as necessary but not sufficient for predictive fertility following coitus or artificial insemination'. As it is difficult to define male fertility and infertility with precision, we must apply a high degree of rigour to evaluating any test that purports to predict fertilization outcome. A variety of statistical approaches have been applied to the problem including non-parametric approaches, logistic regression models, multivariate analysis and more recently multivariate analysis coupled with three-dimensional data plotting or the use of star plots (also called radar plots) to display complex sets of variables.

In real life, the distributions of semen parameters for fertile and infertile men overlap considerably and in addition are usually skewed or otherwise vary widely from the normal distribution, as seen in Fig. 1 (Boyers *et al.*, 1989).

The value of a predictive test can be assessed by comparing the distribution of normal and abnormal individuals for the parameter being measured (Fig. 2). Here the analogy that infertility is a disease state or affliction can be used, and the test has an arbitrary cut-off point that is varied in trying to discriminate between the afflicted and the non-afflicted groups. Thus a 'positive' test identifies

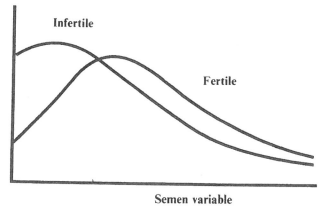

Fig. 1. Typical distribution of a semen parameter (in this case, sperm count) for fertile and infertile men. (Adapted from Boyers *et al.*, 1989 from data of Macleod, 1951.)

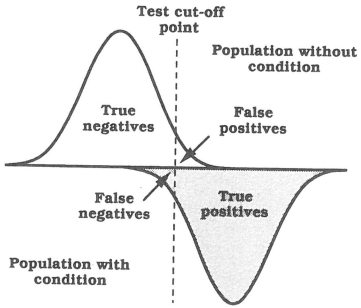

Fig. 2. This demonstrates the near inevitable overlap that occurs in the incidence of a clinical condition, such as infertility, when one applies a given test to a single measure of the condition.

those who exceed the cut-off point: these may be really afflicted ('true positives') or they may be normal, wrongly characterized as afflicted ('false positives').

From Fig. 2 five major descriptors of the test's effectiveness can be derived as shown in Table 1.

No cut-off for any such test can obviously ever perfectly distinguish between the two groups. The fertile and infertile populations generally overlap in any measured parameter (e.g. sperm count, percentage 'normal' forms). Therefore the aim must be to derive the maximum discriminating power by minimizing both false negatives and false positives. A powerful way of demonstrating this is by using 'receiver–operator–characteristic' curves in which we plot sensitivity against false positive rate. Sensitivity and specificity are the most stable attributes of a diagnostic test, as they do not change when the group sizes studied differ. However, as different cut-off values are used, they fluctuate against each other, and the receiver-operator curve is one way of plotting this. A useful test shows high true positive rates while false positive rates are minimal. This is illustrated in Fig. 3.

Treatment of male infertiliby by assisted reproductive technology

Under normal circumstances, semen is deposited in the upper vagina and sperm make their way through the female tract to contact the oocyte (Chapter 8). In sub-fertile men the sperm are deficient in number and/or inherent quality, and the changes of successful fertilization are accordingly reduced or negligible. *In vitro* fertilization (IVF), by bringing the gametes into close apposition, improves the chances of successful reproduction for these men. This was recognized a

Table 1. *Derivations of terms to define a clinical test*

Descriptor	Formula	What does it describe?
Sensitivty (true positive rate)	TP/(TP+FN)	Proportion of afflicted individuals correctly identified as such
False positive rate (=1-Specificity)	FP/(TN+FP)	Proportion of normal individuals incorrectly identified as afflicted
Specificity	TN/(TN+FP)	Proportion of normal population correctly identified as affliction-free
Predictive value	TN/(TN+FN)	Proportion of population with a normal (negative) test who are actually not afflicted
Accuracy	(TP+TN)/(TP+TN+FP+FN)	Overall performance of the test in correct identification of the problem

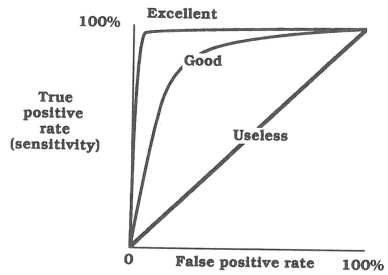

Fig. 3. Derivation of the receiver–operator–characteristic curve.

decade ago and now many men are requesting IVF whereas in previous times they may have been resigned to donor insemination or for the option, now remote, of adoption. This shift in attitude is part of the increased public awareness of reproductive technology and its positive and negative ramifications.

While IVF certainly improves the probability of fertilization, fertilization rates can be disappointing for men with extremely low sperm counts, poor sperm motility or elevated levels of abnormal forms of spermatozoa. Results are particularly bad if all three forms of semen defect are present, or if there are anti-sperm antibodies in the semen. In addition, poor embryo growth rates and quality are observed following the use of low fertility semen. One likely cause of

this is the increased probability that, even if fertilization occurs, it will be late in the fertile life of the oocytes. However, genetic factors may well contribute, as spermatozoa that have been exposed to high levels of free radicals may show increased DNA damage. This is consistent with observations which show that, where embryos are genetically defective, faults due to chromosome breakage largely originate in the spermatozoa whereas gross chromosomal rearrangements are more likely to arise in the oocyte.

The use of metabolic enhancers such as pentoxifylline can improve the chances of fertilization for sub-fertile men, but the long-term genetic consequences of this, while unlikely to be deletrious, are unknown. Other approaches to the management of sub-fertility include increasing the chances of gamete collision by culturing in micro-droplets or capillary tubes. Many IVF groups are now turning to various forms of micromanipulation to enhance fertilization rates (Ng, 1990). This can take the form of drilling or cutting holes in the zona pellucida to enhance the likelihood of poorly motile sperm entering, micro injection of spermatozoa into the perivitelline space, or even injection of spermatozoa directly into the oocyte cytoplasm (Palermo *et al.*, 1993). While there has not yet been any controlled study comparing the use of simple metabolic enhancement with micromanipulation, combinations of the two approaches are being attempted. Some laboratories are starting to use combinations of micromanipulation with metabolic enhancement, coupled with electroporation, to promote acrosome reactions and gamete fusion.

It is hard to predict limits to the possibilities of assisted reproduction. We already know that fertilization can be accomplished with entirely immotile spermatozoa or spermatozoa lacking an acrosome (Aitken, 1990), and there is no theoretical reason why even demembranated sperm nuclei could not be used to generate living embryos. Work from Yanagimachi's group in Hawaii has shown that the nuclei of mammalian spermatozoa are remarkably tough. Isolated nuclei from human, hamster and mouse spermatozoa injected into zona-free hamster eggs can develop into normal-looking pronuclei even after 30 minutes at 90 °C! It is thus entirely possible that isolated sperm nuclei could be stored for long periods without the need for cyropreservation.

The sperm life history

After ejaculation, the spermatozoon passes through a number of distinct phases while interacting with the female tract and preparing for fertilization. Many aspects of this life history are difficult to study *in vivo*, especially in humans, and so we have to extrapolate from studies *in vitro* and from animal models. Fertilization occurs *in vitro* with fairly simple culture media. Thus interaction with the female tract is not obligatory for capacitation. Nevertheless, full

understanding of sperm function requires that we recognize that spermatozoa have been shaped by complex evolutionary pressures including sperm competition in the female tract (Cummins, 1990).

Broadly speaking, sperm movement through the tract involves both passive and active transport phases, during which they enter the cervical mucus and pass into the uterus and then the Fallopian tube. While immotile sperm in some species can pass very rapidly through the tract, it is generally assumed that sperm motility is an important component in both establishing reservoirs of viable sperm in the tract (for example, in the cervix and the uterotubal junction) and in penetrating the various barriers to fertilization. This aspect of sperm function is covered elsewhere in this series. Three ideas are central to the present chapter. These are as follows:

1. Sperm selection occurs at many sites consecutively through the tract, so that the population that reaches the fertilization arena is an 'elite' subset of the original;
2. Tests of sperm function should centre on these selection processes, as they are likely to reveal more about fertilizing potential than simple mensuration of factors in the original unselected population, such as sperm concentration or viability.
3. Several steps in the sequence can be bypassed *in vitro* (e.g. the need to penetrate cervical mucus) but nevertheless can form the basis for useful tests of sperm competence.

Sperm undergo post-ejaculatory changes, collectively known as capacitation, which *in vivo* force a lag phase between insemination and fertilization (Yanagimachi, 1988*a*). Capacitation includes a range of poorly understood processes that do not alter the ultrastructure of the cell but change its potential for fertilization. Changes include sperm surface alterations including the removal of inhibitory 'decapacitation' factors, alterations to sperm intramembranous molecular arrangements, and metabolic changes reflected in a shift to a 'hyperactivated' motility pattern (qv). Some of these surface alterations, for example the binding of Ca^{2+}-conjugated chlortetracycline, can be used to monitor the state of capacitation. They may be useful clinically (see below). The timing of capacitation is variable between species, between individuals within a species and even between spermatozoa within the same ejaculate. It can also be modified by varying experimental conditions. In hamsters, for example, capacitation times can be modulated by elevating the temperature of the sperm store in the cauda epididymis, thus giving some evidence to the idea that capacitation involves a reversal or an 'escape' from the stable state imposed by prolonged epididymal storage.

Even fertile men show variations in capacitation rates *in vitro* as measured by the penetration of zona-free hamster ova with time. These variations are not normally taken into account when preparing sperm for IVF, but perhaps they should be. The major obstacle to doing so is the lack, so far, of any simple and inexpensive method of assessing capacitation rate *in vitro* prospectively, but perhaps the tests of motility or surface changes described below will open up this area.

Hyperactivation

As competent sperm become capacitated they may enter a high-energy phase of exaggerated motility, known as hyperactivation to distinguish it from the *activation* of motility that occurs at ejaculation or when epididymal sperm are experimentally diluted with culture media (Yanagimachi, 1981). This phase of movement has high-amplitude asymmetric flagellation, increased tail curvature and energy output and low linearity in culture medium (Burkman, 1990). Individual spermatozoa can move in and out of hyperactivation if observed over time, but we know little of the energetics or metabolic control of this phenomenon. Presumably hyperactivation assists sperm *in vivo* in swimming through the complex environment of the Fallopian tube and the cumulus matrix. In hamsters, spermatozoa probably use linear motion to pass through the utero-tubal junction and then switch to hyperactivation motility as they enter the ampulla. *In vitro* modelling using hamster sperm exposed to high viscosity medium supports the idea that hyperactivated motility conveys a selective advantage (Suarez *et al.*, 1991). Hyperactivation enabled sperm to generate greater forces in viscous as compared with non viscous media, whereas non-hyperactivated sperm showed similar force output at all viscosities.

The acrosome reaction

The acrosome reaction of the fertilizing spermatozoon, in most species that have been studied, occurs very close to, or on, the zona pellucida. There is some variation in this, however. Guinea pig spermatozoa can only penetrate the cumulus oophorus after they have completed the acrosome reaction. Hamster spermatozoa appear to be limited in their ability to enter the cumulus, to a brief period of capacitation just before the acrosome reaction *per se* (which occurs spontaneously in most sperm after about 4 hours of culture *in vitro*). The evidence for human spermatozoa is equivocal, as a low rate of spontaneous reactions is seen in free-swimming capacitated spermatozoa. However, most authorities agree that there is probably a need for close synchrony between cumulus penetration,

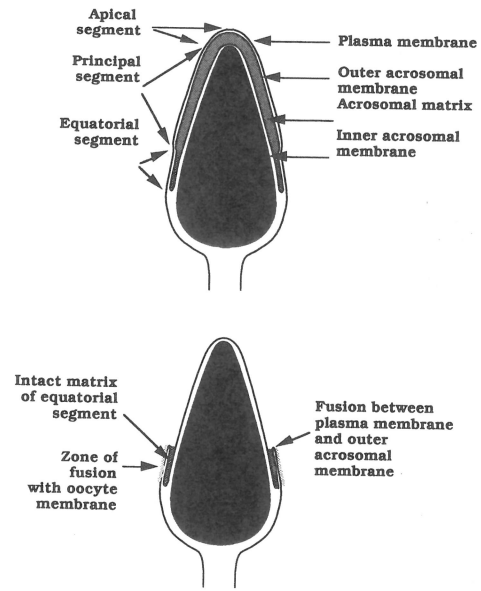

Fig. 4. General features of the human sperm acrosome before and after the acrosome reaction.

approach to the zona pellucida, and completion of the acrosome reaction. Sperm need to be capacitated to achieve this. The biochemical control of the reaction has been best characterized using mouse gametes, where there is a clear distinction between the 'spontaneous' and the 'physiological' (i.e. normal) reaction on the zona pellucida (Storey, 1991). A specific protein component of the zona (ZP3) is responsible for binding the spermatozoon and initiating the acrosome reaction. This is covered in detail below in the section dealing with sperm–zona binding. The binding event probably triggers a membrane fusion cascade involving Ca^{2+} influx, guanine nucleotide-binding ('G') proteins, protein kinase C and phospholipase C. The consequence of this is a process of point fusions between the plasma membrane and the outer acrosomal membrane resulting in pores that permit the acrosomal contents to escape. These processes are summarized in Figs. 4 and 5.

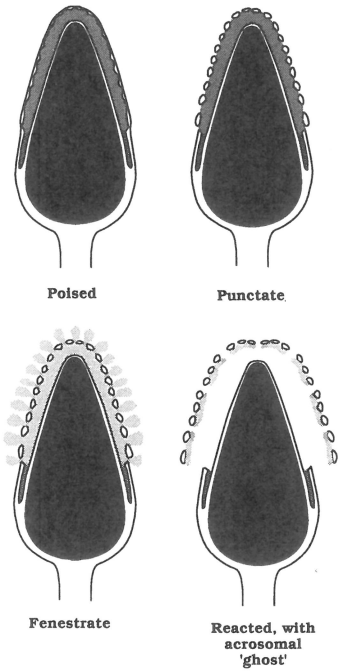

Fig. 5. Sequence of events during the acrosome reaction (after Storey, 1991 and others).

The acrosomal matrix contains a number of enzymes of which probably the most important is the serine protease acrosin (packaged as the inactive precursor proacrosin). Other hydrolytic enzymes include hyaluronidase, neuraminidase, acid phosphateses and esterases.

In the physiological acrosome reaction, membrane fusion only occurs over the principal and apical segments, and the equatorial segment remains intact. At least five separate steps can be dissected out based on chlortetracycline binding patterns in mouse spermatozoa (Storey, 1991) and these are illustrated in Fig. 5. Fusion between the outer acrosomal membrane and the plasma membrane around the head in this region assures cell integrity. From a point of understanding sperm function in fertility and infertility, the following points are important.

1. The physiological acrosome reaction only occurs in capacitated spermatozoa upon binding to a suitable ligand. In the mouse this is ZP3, but in other species a variety of alternative ligands can elicit a reaction, including progesterone, follicular fluid, and a variety of glycosaminoglycans such as heparin. The proportion of spermatozoa that can respond to a ligand varies widely according to the species, to the individual, and to the capacitation culture conditions. I will argue below that variation in this proportion is the basis for differences in fertilizing capacity between different sperm populations.

2. In capacitated human spermatozoa a reaction can be induced by treating with divalent cation ionophores such as A23187 or ionomycin. Again, wide differences between individuals in the proportion of spermatozoa that can respond indicate that this may be one cause of fertilization failure, and I describe below a test of human sperm function based on this.

3. Physiologically, the relevance of the acrosome reaction is that it permits normal penetration of the zona pellucida. It also exposes or activates binding sites to the egg surface over the postacrosomal head region (Yanagimachi, 1988*b*).

Penetration of the zona pellucida

The zona pellucida is one of the most important barriers faced by the fertilizing spermatozoon. The zona is a biochemically complex structure which in the mouse (the best studied model) consists of three acidic glycoproteins, ZP1 (200 kD), ZP2 (120 kD) and ZP3 (83 kD) arranged in a complex three-dimensional matrix. ZP2 and ZP3 are filamentous molecules cross-linked by the globular ZP1. A class of O-linked oligosaccharides on ZP3 may act as the primary receptors for the fertilizing spermatozoon and thereby induce the acrosome reaction through activation of sperm G proteins, phospholipase C and protein kinase C (Wassarman, 1991). There is increasing evidence (in the mouse) that sperm surface molecule that recognizes ZP3 receptors is a species-specific glycoside transferase enzyme that recognizes the binding oligosaccharides on ZP3. Almost certainly the oligosaccharides will give sperm–zona binding its specificity. In human fertilization binding appears to be mediated by D-mannose. The plasma membrane contains D-mannosidase and sperm of fertile men cultured under capacitating conditions show a characteristic increase in D-mannose binding activity and pattern that is not seen in infertile men (Tesarik, Mendoza & Carreras, 1991).

Following attachment, the acrosome-reacted spermatozoon remains bound to the zona pellucida, by specific receptors to ZP2 localized on the inner acrosomal membrane. Penetration of the zona matrix by the fertilizing sperm is not well understood. Only a narrow penetration slit is formed. It presumably involves a combination of physical forces driven by the hyperactivated motility of the sperm

flagellum acting through the sharp edge of the tip of the head, and limited proteolytic cleavage (Oehninger, 1992). Following successful fertilization, release of cortical granule proteases from the activated oocyte (the cortical reaction) results in inactivation of the sperm receptor and acrosome reaction-inducing properties of ZP3. Proteolytic changes occur in ZP2 and the zona pellucida 'hardens'.

This secondary block to polyspermic fertilization (the primary one being at the oocyte surface) is an essential component to maximize the chances of normal syngamy. Not surprisingly, the zona pellucida in most cases has a high degree of species-specificity for sperm binding and penetration. While commonly cited as being a barrier to inter-species fertilization, this specificity is probably more a result of drifting molecular mechanisms, in response to the intense sexual selection pressures on fertilization mechanisms. Inter-species mating leading to gamete interaction is not common (Cummins, 1990). The human zona pellucida also contains a group of three glycoproteins, although the role of ZP3 in particular in inducing the acrosome reaction is not as clear-cut as it is in the mouse. Human sperm acrosome reactions can be induced by a wide variety of the molecules likely to be in close vicinity to the oocyte including follicular fluid, progesterone and components of the cumulus matrix as well as by the zona pellucida. In addition, of course, human spermatozoa cultured under capacitating conditions will show a relatively steady rate of spontaneous acrosome reactions, the level of which is only poorly related to fertile status (Cummins *et al.*, 1991).

Sperm subsets

There is good evidence that spermatozoa fall into a number of population subsets, with different potential for fertility. Conventional approaches to IVF tacitly assume this point, and there is extensive literature on techniques for separating 'good' from 'bad' spermatozoa. There are four basic approaches which Mortimer (Mortimer, 1990) has described in detail. These are simple dilution and washing; sperm migration 'swim up' or 'swim down' techniques relying on inherent motility; selective washing techniques based on density-gradient centrifugation; and selective adherence or filtration techniques such as the use of glass wool or Sephadex®. Several commercially available systems such as the SpermPrep Sephadex columns are now available. For most practical purposes in IVF a simple 'swim up' technique suffices. For poor quality semen, discontinuous Percoll gradients or continuous Nycodenz gradients give superior results. Any technique used should minimize shear forces that can result in free radical release and lipid peroxidation, and in addition should aim at separating the 'best' sperm rapidly from seminal leukocytes. Activated leukocytes are a potent source of radicals

(Aitken *et al.*, 1992*b*; Kessopoulou *et al.*, 1992) and can markedly reduce fertilization rates *in vitro*.

Ideas on subsets in the sperm population have been around for a number of years. Twenty years ago Cohen even suggested that the great majority of mammalian sperm may be dysfunctional through meiotic errors and that the female tract acts to select against all but an 'elite' sub-population that is permitted to reach the site of fertilization. The means of such selection are not clear, but may be immunological. Certainly the numbers reaching the ova are very low: in the hamster, for example, eggs *outnumber* sperms in the ampulla during the early phases of fertilization and similar low gamete ratios exist in other mammalian species. Experiments using IVF with very low sperm–egg ratios or even micro-insemination with operator-selected single spermatozoa have seriously challenged Cohen's hypothesis, and recently Ivani and Seidel (Ivani & Seidel, 1991) have shown that around 50% of capacitated motile mouse sperm chosen at random by micromanipulation can fertilize zone-free oocytes. Conversely, it could be argued that 50% of sperm are *incapable* of fertilizing! Cohen's arguments on genetically programmed dysfunction may be difficult to disprove, but they have been remarkably successful in forcing us to think about the nature of the sperm population and its interaction with the female reproductive system. One must recognize that evolutionary pressures, such as sperm competition between males, drive the form and function of the sperm cell and much of this is still obscure (Cummins, 1990). Recently Eisenbach and Ralt (Eisenbach & Ralt, 1992) have suggested that there is a turnover of fertilizing spermatozoa in the female tract so that at any given moments only a small percentage are able to fertilize. They couple this idea with the possibility that the egg might emit attractants, but the evidence for true chemotaxis in mammalian spermatozoa (as opposed to chemokinesis or 'trapping' by changes in linearity of motion) is not very convincing.

Evidence accumulating from a number of areas, therefore, suggests that only a sub-set of human sperm is fertile, and that many sperm are dysfunctional. The differences between fertile and infertile men may thus lie in the relative proportions of functional versus dysfunctional spermatozoa that can be produced, and tests of sperm function are likely to be most helpful if they are aimed at elucidating this proportion. This idea is summarized in Fig. 6. Man may indeed be fertile, despite very low sperm counts, provided the sperm that are produced are fully competent. This is typically seen in men with gonadotrophin deficiency when testicular function is rescued with exogenous hormone treatment. Thus research on the inductibility of the acrosome reaction referred to below indicates that in subfertile men few or none of spermatozoa cultured under capacitating conditions can respond to calcium influx by undergoing the acrosome reaction (Cummins

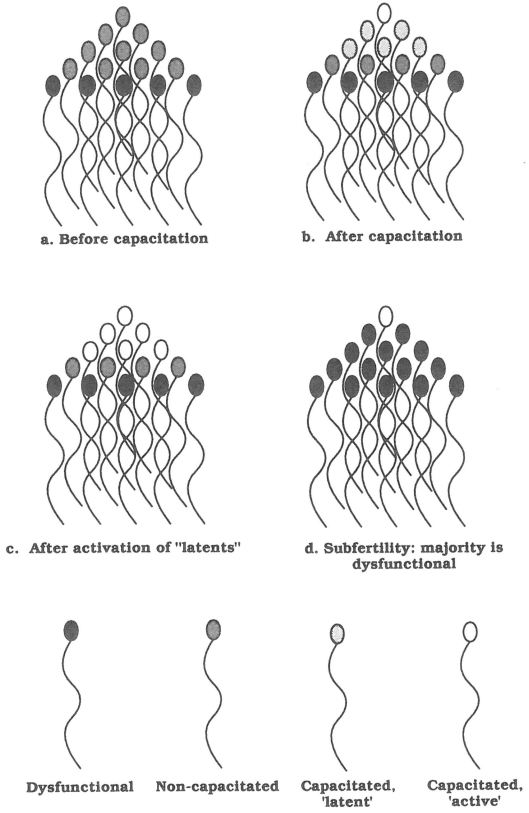

Fig. 6. This is a schematic depiction of the idea of sperm subsets, in fertility and infertility. Dead spermatozoa are not considered here. In (*a*) the sperm population consists of a mixture of dysfunctional and non-capacitated cells. In (*b*) a period of capacitation with a fertile population results in a mix of non-capacitated and capacitated spermatozoa. Of the capacitated set, only a few are 'active' in that they exhibit hyperactivation and (possibly) spontaneous acrosome reactions: the rest are capacitated but 'latent', awaiting a suitable stimulus. In (*c*) a stimulus (such as A23187) has been applied, exposing the total 'latent' population. I suggest that, in the case of a subfertile man, the majority of sperm are dysfunctional and cannot even enter the 'latent' phase of capacitation. This results in (*d*).

et al., 1991). Likewise, work on the distribution of creating kinase isoforms in human spermatozoa demonstrates a strong association between the immature B-form and infertility (Huszar, Vigue & Morshedi, 1992). The presence of this enzyme isoform may be a marker for residual sperm cytoplasm resulting from imperfect spermiation, and such abnormal sperm forms, along with seminal leucocytes, are more likely to release the damaging reactive oxygen species that are thought to be implicated in poor sperm function.

The idea of subsets opens the way to sperm separation. Sample enrichment could be based on expression of a surface marker, or specific pattern of a marker for capacitation such as chlortetracycline (below). In animal breeding, preliminary reports show that flow cytometry can successfully identify sub-populations, monitor changes in semen quality and separate sexed sperm for IVF or artificial insemination (Morrell, 1991). Human sperm sex chromatin can be detected in decondensed nuclei using fluorescence *in situ* hybridization with a DNA probe specific for the Y chromosome. It has not yet been possible to apply such probes to living, condensed nuclei but the eventual application of similar techniques to human reproduction seems certain.

Indirect tests of sperm function

Conventional semen parameters

While I have argued above that conventional sperm counts are of limited value in predictive fertility, there is no doubt that good semen analysis, and investigation of any possible underlying disorder, must be performed as part of routine investigation. The nature of routine semen analysis is covered by exhaustive reviews elsewhere (Mortimer, 1990) and need not be repeated in detail here. However, at minimum it should include observation of physical parameters such as liquefaction, pH and volume together with cell concentration, morphology and viability (WHO, 1987; 1992). In addition, the laboratory should assess the presence of auto immune antibodies to spermatozoa, in both male and female partners. These have the potential to inhibit fertilization possibly by inhibiting the acrosome reaction. However, the role of antibodies in IVF failure is complicated. Major antigenic differences may exist between capacitated and non-capacitated spermatozoa, as cases are known where women have developed antibodies against the former but not the latter.

Nuclear decondensation in vitro

Under normal circumstances the sperm nuclei become condensed during the final stages of spermatogenesis. Cysteine-rich protamines replace histones. Further

hardening of the nuclear matrix, as well as alterations to the cytoskeletal elements of the flagellum, occurs by increasing disulphide bonding during epididymal maturation. However, the transit time for sperm through the human epididymis is enormously variable and may be indeed only a few days in men with concurrently impaired spermatogenesis (Johnson & Varner, 1988). Variations in the degree of nuclear condensation can be evaluated by acridine orange fluorescence staining which identifies 'immature' or imperfectly condensed DNA. The use of acridine orange staining to evaluate human spermatozoa clinically shows a high level of correlation between normal DNA and 'normal' morphology, but strict morphological assessment would appear to be a better predictor of fertilization *in vitro*.

It is essential that rapid nuclear decondensation occurs following sperm entry to the oocyte, otherwise correct syngamy will fail. Human sperm heads can be decondensed *in vitro* relatively simply using combinations of detergents and disulphide bond reducing agents such as dithiothreitol. At least one report has suggested that this propensity is linked to fertility and thus could serve as a simple predictive laboratory test (Chan & Tredway, 1992) similar to the hypoosmotic swelling test described below.

Hypoosmotic swelling test

Living sperm normally actively exclude water by osmoregulation, but if suspended in hyopoosmotic solutions this capacity is overcome and they will undergo swelling of the tail membrane. This is reversible provided only the principal piece and not the midpiece is involved. The hypoosmotic swelling test exploits this capacity (Jeyendran *et al.*, 1984) and in essence evaluates the functional integrity of the sperm membrane. The correlation between hypoosmotic swelling and fertilizing capacity is not especially good but the technique is simple and easy to quantitate and it has been used in combination with tests of acrosomal function to combine information about capacitation status and viability simultaneously.

Axonemal ultrastructure

Poor sperm motility is undoubtedly associated with infertility, and motility defects have been linked with underlying abnormalities in axonemal structure (Serres, Feneux & Jouannet, 1986). Even fertile men show considerable heterogeneity in axonemal structure. Due to the great technical difficulties and time involved in sectioning and analysing sperm ultrastructure, it is unlikely that this approach will ever be used for routine prospective appraisal. However, ultrastructural studies are of undoubted immense value for diagnostic purposes where a structural defect is suspected.

Measurement of acrosomal enzymes

The acrosome reaction is essential for normal penetration of the zona pellucida and the neutral protease acrosin is thought to be the major one responsible. Acrosin is packaged in the form of an inactive zymogen, proacrosin and there is indirect evidence that conversion of proacrosin to acrosin in human spermatozoa may precede the membrane fusion events of the acrosome reaction. Conversion of proacrosin to acrosin and then subsequent inactivation following limited proteolysis of the zona pellucida has been proposed as a mechanism of zone penetration that could explain the very limited extent of lysis that occurs in the penetration slit. The measurement of acrosin/proacrosin levels in semen may provide a valid measure of sperm competence, and this would certainly be a way of establishing total absence of acrosomes.

The gelatine substrate test is a simple way of demonstrating the presence of proteases in the acrosome. In this test, spermatozoa are washed and spread onto a thin layer of gelatin on a glass slide. Following incubation in a humid incubator, proteases leach out of the acrosome and cause a 'halo' of dissolved gelatin to appear around the sperm head. After fixation this can be seen easily using phase contrast microscopy, and the size of the halos together with the number of spermatozoa with halos can be quantitated. 'Dead' sperm that have lost their acrosomes do not develop a halo. This has the attraction of being both cheap and simple, but its relevance to fertility has not been firmly established.

Measurement of seminal ATP content

Much of sperm function depends on the production of ATP and ATP content has been proposed as a measure of fertility. However, the relationship is obscure as motility and sperm function are governed by a complex of factors including cAMP, adenosine, calcium ions and pH (Hoskins & Vijayaraghavan, 1990). Certainly the regulation of intracellular ATP levels is closely coupled with motility, as about 50% of the intracellular ATP pool is metabolized each minute (Ford & Rees, 1990). However, total seminal ATP may not necessarily reflect that available for motility or for fuelling the acrosome reaction as there is evidence (discussed again below) that high energy phosphate bonds from mitochrondrial ATP are passed to the flagellum in the intermediate form of creatine phosphate: the 'creating–creatine phosphate shuttle' (Thombes & Shapiro, 1985). Thus the measurement of total ATP is unlikely to be of more than indirect clinical interest in predicting fertility. A recent report from the World Health Organization (1992) confirms that neither ATP content nor conventional sperm parameters have any predictive value for fertility when the sperm count is 'normal' (>20 million per ml).

Other biochemical tests

Several biochemical tests have been proposed as indicators of sperm dysfunction. Thus creatine phosphokinase (CK), the major enzyme thought to be responsible for moving high energy phosphate groups from the midpiece mitochrondria to the axoneme in the 'creatine shuttle' (Tombes & Shapiro, 1985), exists in two isoforms, the 'B' (brain-type) isoform characteristic of immature cells and tissues, and the 'M' (muscle-type) isoform characteristic of mature or differentiated cells. Huszar and his co-workers have demonstrated a clear relationship between a high B:M isoform ratio and infertility (Huszar *et al.*, 1992). It is not yet clear whether the high level of B isoform CK is directly related to sperm dysfunction (for example, by affecting the movement of high energy phosphate groups between mitochondria and the axoneme) or whether it is simply a marker for poorly differentiated cells. Other potential biochemical markers for sperm abnormalities include the sperm-specific isoenzyme for lactate dehydrogenase (LDH_x) and glutamic-oxaloacetic acid leakage; but, as Mortimer has pointed out, the clinical significance of these markers remains to be established (Mortimer, 1990).

Computer-aided semen analysis

Computer analysis falls into two broad areas. Sperm motion analysis coupled with semi-objective evaluation of concentration is commonly called CASMA (Computer aided sperm motion analysis). Automated sperm morphology analysis is also evolving, but is currently less well developed.

The development of computerized counts and motion analysis has greatly changed the way in which we can apply understanding of sperm motion to the study of fertility. The topic is the subject of exhaustive and authoritative recent reviews and a full evaluation is not strictly relevant here (Boyers *et al.*, 1989). The instruments that are currently commercially available evolved historically from techniques such as time-exposure or multiple-exposure photography that provided an overlapping series of sperm head images that allowed tracking of sperm movement on a two-dimensional basis. In the mid-1980s the development of high speed cinematography and videomicrography coupled with digitized analysis and storage of coordinates permitted more sophisticated investigations of sperm movement. Parallel developments in image analysis in areas such as tomography and cytology allowed better analysis of sperm head shape for toxicology as well as in the investigation of infertility. Today, a number of commercial units are available and much effort is being devoted into developing suitable software and standardizing operating conditions so that results can be compared within and between laboratories. Despite these rapid developments, it

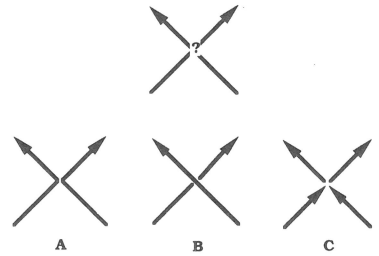

Fig. 7. This illustrates the 'bump and cross' problem in interpreting sperm tracks using computer-aided motion analysis. Intersections have a 'grey area' where the size of the overlapping heads falls outside the set defined as 'normal' for a single sperm head. Depending on the algorithms used and the definition of the 'best' fit for the available data, the computer may interpret the intersection as: A two sperm that zig-zag back on themselves; B correctly as two sperm intersecting; or C as four separate sperm tracks.

does not seem that computers can yet offer completely automated and error-free systems. Most reviewers agree that interpretation of results must be validated by expert observation. Videomicrography offers the advantage of immediate recording and multiple replaying. High-speed microcinematography, while currently capable of better resolution and higher observation frequencies, is expensive and time-consuming and therefore not suitable for routine andrological investigation.

The major inadequacies in present systems include low field rate observation frequencies, typically 30 to 60 fields per second, whereas sampling rates of at least 120 fields per second are needed to fully characterize the harmonic behaviour (oscillation frequencies) of human spermatozoa. Even higher frequencies may be needed for hyperactivated sperm or very rapidly swimming forms. Russell Davis, from the group at David, California, recommends a minimum of 200 frames per second for monkey spermatozoa (Davis *et al.*, 1992). Other problems not yet fully vanquished include the 'bump and cross' phenomenon, which occurs when sperm paths intersect and the computer is unable to resolve which sperm is emerging from the intersection. Different configurations deal with this problem in different ways. At least one system ignores all 'bump and cross' paths and does not include them in any analysis. This, however, introduces bias at higher sperm concentrations and swimming speeds when intersections are more likely to happen. Other systems look for the 'most likely' next position, but it is easy for the computer to be fooled and to identify the wrong paths. Fig. 7 demonstrates a variety of possible outcomes. In addition, problems are caused by sperm agglutination, surface effects such as 'spinning', cells entering or leaving fields, out-of-focus artefacts, and factors

affected by sperm density. There is, yet, no fool-proof system, and there is no substitution for critical evaluation of all recorded paths to eliminate such artefacts.

Thus, although computer systems have been widely advertised as being suitable for routine andrology, at least one recent review recommends the user first to perform manual concentration estimations, by traditional means (haemocytometer or other counting chamber), and then adjust the concentration to <50 million per mil for motility analysis (Davis, 1992)! Despite these caveats, there is no doubt that, provided rigorous attention is paid to recommended standard procedures (Davis, 1992), good computer analysis will give more accurate results than manual equivalents. As experience accumulates in the use of these systems, advanced algorithms for evaluating sperm motion are emerging which give more accurate measures of sperm behaviour than those currently available commercially.

While most current effort is directed toward systems for tracking sperm head motion there is no doubt that future generations of computer systems will allow us to monitor the much more complex movements of the flagellum. A number of studies of flagellar beat frequency, amplitude, wavelength, rotation frequency together with progressive velocity and power output have already been accomplished for mammalian spermatozoa. Stephens and Hoskins have described a computerized semi-automatic system that evaluates flagellar form (Stephens & Hoskins, 1990). Future developments will probably also allow the three-dimensional analysis of sperm swimming free of surface effects. However, limitations in computer memory space and in the capacity to deal with the enormous volumes of data that are generated means that such systems are far from being in routine clinical use.

Turning to automated morphology assessment, several commercial systems offer fairly simple assessment of head shape based on videomicrography. These systems measure factors such as head length, width, the head perimeter and area ellipticity (defined as (length − width)/(length + width)) and form (defined as 4π area/perimeter^2). In addition, systems can report on staining characteristics such as the amount of light transmission and absorbence and overall dye staining (defined as area × absorbence). Katz (Boyers *et al.*, 1989) has proposed a standardized set of descriptors as shown in Table 2. This can be used to produce a template defining 'normal' head shape limits (Fig. 8).

Classification systems such as these have been applied to the study of reproductive toxins and related to motility and fertility (for review see Boyers *et al.* (Boyers *et al.*, 1989)). At least one commercial system ('Morphologizer' supplied by Cryo Resources, NY) will 'learn' customised classification systems, and more advanced research systems using grey-scale image processing combined with multivariate analysis have been published. No system yet appears to deal with the question of midpiece or tail abnormalities, even though these are significant for understanding impaired motility and infertility.

Table 2. *Techniques for staining the human sperm acrosome*

Technique	Target	Advantages	Disadantages
Triple-stain	Differential staining of acrosomal matrix	Uses light microscopy: incorporates live–dead stain	Inconsistent stain characteristics
FITC-Peanut (PNA) lectin	Outer acrosomal membrane	Easy to use	Small target size. Plasma membrane needs to be permeabilized: false positive results possible
Ricinus communis lectin	Acrosomal matrix	Easy to use, large target size	Extremely toxic lectin; ethanol permeabilization can give false positive results
FITC-*Pisum sativum* (PSA) lectin	Acrosomal matrix	Large target, ease of distinguishing acrosomal regions, easy to apply	Ethanol permeabilization may increase false positive results
FITC-concanavalin A lectin	Inner acrosomal membrane	Highly specific: aldehyde fixation minimises artefacts and 'false' reactions	Small target size
Indirect immuno-fluorescence using monoclonal antibodies (MABs)	Wide variety of ligands depending on MAB	Highly specific; can be used to trace movement of antigens during AR (e.g. PH20 in guinea-pig sperm	Requires access to MAB library

Direct function tests

Penetration of cervical mucus

Cervical mucus is normally the first selective barrier experienced by the spermatozoon in the female tract, and appraising the interaction between sperm and mucus should form part of every infertility investigation. Cervical mucus is a complex sialylated mucin secreted by non-ciliated epithelial cells of the cervical crypts. It is an elaborate micro-environment for the spermatozoon. It consists of

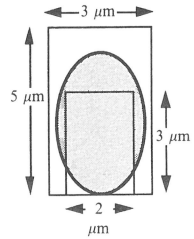

Fig. 8. This illustrates a template for judging correct head morphology. 'Normal' oval sperm head maximum width and length should lie between the two box outlines. (Adapted from Katz *et al.*, 1981.)

an aqueous phase containing serum exudates and other secretions, and an insoluble gel phase consisting of a sialoglycoprotein matrix. This complexity lends cervical mucus, like other epithelial mucins, complex physical properties such as viscosity, spinnbarkeit ('stringiness'), elasticity and the demonstration of crystalline 'fern' patterns upon air drying. These several components show marked variation throughout the menstrual cycle in response to the changing endocrine milieu and evaluation of these physical properties gives a valuable test of the stage of the menstrual cycle at which the cervix is most (or least) receptive to sperm penetration. In addition, presence of antisperm antibodies or other 'hostile' factors can impede sperm transit and form one cause of infertility. The interaction of sperm with cervical mucus *in vitro* is complex due to the changing microstructure of mucin strands as the mucus is manipulated by stretching or shear forces.

First anticipated 60 years ago, *in vitro* tests of sperm interaction with cervical mucus either on glass slides or in capillary tubes have proven invaluable. The test was placed on a standardized practical and theoretical basis by Katz's group at Davis, California, over a decade ago (Katz, Overstreet & Hanson, 1980) and several studies have now shown that the ability of spermatozoa to penetrate cervical mucus is correlated with specific movement characteristics and with the ability of sperm to fertilize both *in vitro* and *in vivo*. To be valid, the test should be run as a cross-over trial for infertile couples: husband and fertile donor sperm should each be tested against the wife's and fertile donor mucus. However, this is logistically difficult to set up and the use of frozen mucus or mucus analogues is probably more practical (below).

Aitken has shown that the degree of lateral head displacement demonstrated by free-swimming spermatozoa is the most important single motion parameter

that determines the success of penetration (Aitken, 1990). This high amplitude beat is one important characteristic of the sperm movement that increases during hyperactivation. It probably reflects the energy output of the sperm cell. There is a complex interaction between sperm mucus penetration and capacitation that is only poorly understood. Sperm that have passed through a mucus column appear to be capacitated in that they will penetrate zona-free hamster eggs very rapidly (Katz, Andrew & Overstreet, 1989) and will show increased capacity to acrosome reaction following a suitable stimulus such as exposure to the zona pellucida, or follicular fluid. However sperm recovered from the cervical mucus reservoir as long as 72 hours after coitus show no increase in the rate of spontaneous acrosome reactions. Most likely a combination of physiological interaction, physical filtering of 'good' from 'bad' sperm and suppression of the acrosome reaction is at work.

Penetration of mucus analogues

The major problem in using homologous cervical mucus as a routine test system is the difficulty of obtaining and storing mid-cycle mucus of standard physico-chemical composition. This poses severe obstacles to standardizing the test, particularly in multi-centre and cross-over trials. Alternatives to human mid-cycle mucus are now available commercially. Thus Penetrak® is a preparation of bovine mid-cycle mucus supplied frozen by Serono Diagnostics (Wellyn Garden City, UK). A purified Hyaluronate polymer gel, Sperm Select®, is also available from Pharmacia (Uppsala, Sweden). Aitken has shown (Aitken *et al.* 1992*a*) excellent correlations between penetration of the two preparations. Penetration of sperm into Sperm Select® was slightly better than Penetrak® in accounting for the results observed in an ionophore-enhanced zona-free hamster ovum penetration test (see below). The Sperm Select® test was capable of successfully identifying the group of patients in whom zero penetration occurred in the egg penetration test. Other proposed analogues for cervical mucus include egg white and serum albumen but these do not appear to have been evaluated in any systematic manner.

Hyperactivation assays

The development of computer assisted motion analysis opens the way for diagnostic tests of human sperm capacitation as manifest by the onset of hyperactivation. Burkman (1990) has defined the parameters by which hyper-activated spermatozoa may be discriminated from non-hyperactivated using computer analysis. In essence, sperm defined as hyperactivated show a simultaneous

combination of high curvilinear velocity (>100 μ/second), high beat amplitude (>7.5 μ) and low linearity (<65%). The dynamics of spontaneous onset of hyperactivation, in sperm from fertile donors cultured under capacitating conditions, show a close relationship to those of spontaneous acrosome reactions. There are no reports yet linking hyperactivation with fertility as a predictive test; however, this approach seems very likely. We have observed (unpublished observations) that metabolic enhancers such as pentoxifylline stimulate high levels of hyperactivation for normospermic samples for about 2–4 hours, and markedly enhance the rate of acrosome reactions in response to A23187. One could possibly use such techniques to determine the proportion of sperm in the population capable of undergoing hyperactivation. This may offer an attractive way of testing fertile potential, by deliberately challenging the sperm population to demonstrate its maximum capacity for hyperactivation. A hyperactivation motility assay requires only 5–10 μl of dilute sperm suspension, so that the sperm preparation that is used for IVF could be monitored simultaneously for hyperactivation status and potential. We are currently evaluating this approach as an alternative to the ARIC test described below.

Acrosomal function assays

Completion of capacitation and initiation of the acrosome reaction on or close to the zona pellucida are essential steps in fertilization. There is good evidence that failure in one of these steps is an important cause of infertility. Thus Byrd and Wolf (1986) showed that a significant subset of sperm from infertile men cultured under capacitating conditions do not respond to the calcium ionophore A23187. In oligozoospermic men, A23187 treatment does not enhance sperm penetration of zone-free hamster eggs (below), and although this can be increased by treatment with follicular fluid, there is no increase in the proportion of acrosome reacted sperm. The cause of this failure is not clear, but it is likely that the spermatozoa have either suffered damage due to free-radical-induced lipid peroxidation or are somehow incapable of completing the normal capacitation sequence.

Studying the human sperm acrosome has been difficult until fairly recently. It is relatively thin and overlies a sperm head that varies in thickness, being spatulate distally but oval in cross-section in the middle. This gives the sperm head an oval profile but a wedge-shaped sagittal section. This varying optical thickness makes the components of the human sperm head difficult to evaluate by conventional microscopical techniques such as phase contrast, that can be applied very readily to the study of acrosome reactions in species such as the hamster and guinea pig. This made study of the dynamics of the acrosome reaction difficult.

The small size of the acrosome also means that vital stains such as acridine Orange, used to differentiate the acrosomes of sperm of laboratory animals cannot be applied to human spermatozoa. The first major technical breakthrough came when Talbot and Chacon (1981) developed a triple-stain approach. This not only identified the acrosome but also included a vital stain (Trypan blue) that was incorporated by 'dead' (membrane permeable) cells. However, the technique had a number of difficulties, both in standardizing stain between-batch variations, and in applying it readily to experimental and clinical situations.

The development of fluorescently labelled lectins and specific antibodies together with other membrane stains such as chlortetracycline greatly simplified matters. We now have a variety of staining protocols that can be applied alone or in combination with vital stains such as Hoechst 33258 to distinguish 'live' from 'dead' sperm. An alternative is the combination of lectin staining with the hypoosmotic swelling test that likewise will clearly indicate whether acrosome reactions are occurring in spermatozoa with functionally competent plasma membranes. Table 2 gives a summary of the major techniques, their staining target(s) and their major advantages and disadvantages.

In studying the acrosome reaction, it is important to distinguish between spontaneous reactions and those which suitable stimuli can elicit in capacitated fertile spermatozoa. Almost certainly, the physiological reaction in human sperm accompanies the final stages of approach to the oocyte through the cumulus oophorus. Human sperm with intact acrosomes readily penetrate the cumulus *in vitro* and the reaction rate seen *in vitro* probably bears little or no relationship to fertile potential. This is very different from species such as the hamster or the guinea pig where most spermatozoa will undergo spontaneous reactions. Tesarik (Tesarik, 1989) analysed this question carefully considering other tests currently available such as the hamster ovum penetration test (qv) and concluded that, 'the only physiological acrosome reaction is that produced by an appropriate biological stimulus, while spontaneous acrosome reactions have little biological efficiancy'. He concluded that tests of sperm function based on acrosome reaction potential 'should therefore be designed to distinguish between the spontaneous and induced acrosome reactions'. Working independently, my colleagues and I had arrived at the same conclusion. We decided to use the calcium ionophore A23187 (10 μM) to induce Ca^{2+} influx in a sperm population prepared under capacitating conditions identical to those used for IVF. Besides the ionophore-treated spermatozoa we treat a control aliquot with vehicle alone (10% DMSO in protein-free culture medium). The vehicle has no significant effect on acrosome reactions. After one hour, we wash the sperm through 60% Percoll and then fix and permeabilize the sperm with 95% ethanol. The sperm are spread onto glass slides and stained with FITC-labelled *Pisum sativum* lactin. This binds to the

acrosomal matrix. Sperm with an intact acrosome show complete and uniform staining over the anterior head. Sperm that have completed the acrosome reaction show a belt of staining representing the equatorial segment. Intermediate stages can also be distinguished, and sperm that were degenerate at the time of fixing generally show no staining at all. This is because the equatorial segment is relatively transient and starts to break down soon after the sperm dies or completes the acrosome reaction. The sperm are examined using fluorescence microscopy and classified according to acrosomal status.

We use the difference between the A23187-induced reaction rate and the background spontaneous (control) rate as the ARIC value (acrosome reaction following ionophore challenge) (Cummins *et al.*, 1991). We arrived at the following characteristics for the test (Table 3), based on 53 fertile and 26 sub-fertile men. The men were characterized on the grounds of their proven fertility in IVF. Sub-fertility was defined as less than 50% fertilization rate when four or more oocytes were inseminated and no other likely cause of the poor fertilization could be identified.

As can be seen, an ARIC cut-off of 5% has a high predictive value (90%) and is highly specific (98%) while giving a false-positive rate of only 2% (only one of our group of 53 controls fell in this range). We have now screened several hundred men using this test and the results are highly consistent. Men presenting with a test result of less than 5% are counselled about the likelihood of poor fertilization in IVF.

The test thus seems to be broadly comparable to the hamster ovum penetration test, described below, but is much cheaper and quicker taking at most 3–4 hours. We have been unable to compare it directly with the hamster egg penetration test as hamsters are forbidden laboratory animals in Australia. Work coming from several laboratories (Japan, Chile, UK) is now beginning to confirm that the two tests are very similar clinically. The major disadvantage of the ARIC test as currently applied is that it requires at least 2×10^5 motile sperm per ml following sperm preparation. For normospermic men this is not a problem but for severely oligoospermic men we are, occasionally, unable to recover enough motile spermatozoa for evaluation. In these cases, of course, the men will have severe difficulties in producing enough sperm for conventional IVF and may need to move to alternative approaches such as micromanipulation. We have attempted to use a micro-method for evalating acrosomal status based on preparing small sperm numbers on a filter, but without much success.

While we have found the ARIC test to be clinically useful, ionophores such as A23187 can give varied results according to protein type and concentration and the underlying metabolic state of the cell. In addition there may be batch variations and other preparative problems (e.g. photosensitivity) with ionophores. The free

Table 3. *Test characteristics for various cut-off levels of the ARIC test in distinguishing between fertile and sub-fertile men*

ARIC threshold (%)	3	5	10	15
Sensitivity (%)	23	35	54	69
Predictive value (%)	100	90	64	56
Specificity (%)	100	98	85	74
95% confidence – Intervals of specificity		95.6–100	76.9–93.5	63.2–84.0
False-positive rate (%)	0	2	15	26

From Cummins *et al.*, 1991.

acid is recommended as it shows less batch variation than the Ca^{2+}/Mg^{2+} salt, however it also shows quite strong autofluorescence that can be a disadvantage in some physiological studies (Ford *et al.*, 1991). It is essential that each laboratory using this approach titrates ionophore concentrations carefully using known fertile donor spermatozoa to ensure that only the mimimum amount of A23187 (or ionomycin) is used to stimulate a maximal acrosome reaction response. Future developments in the field may well rely on different modes of stimulating the acrosome reactions: for example, the use of fusogenic phospholipid liposomes.

Use of follicular fluid to stimulate the acrosome reaction and/or fertility ability

Various workers have proposed the use of human follicular fluid to maximize the acrosome reaction or to stimulate fertilizing ability. While follicular fluids have marked effects on sperm motility and can stimulate acrosome reactions very rapidly, within a few minutes, (Yudin, Gottlieb & Meizel, 1988) they are highly variable in this capacity. This, presumably, reflects heterogeneity in the physiological status of follicles following ovarian stimulation. This variability is due in part to the varying progesterone content of the follicular fluid. Progesterone provokes a rapid Ca^{2+} influx and will result in the acrosome reaction in competent capacitated spermatozoa, and there is some evidence from Meizel's group in California that this steroid acts through $GABA_A$ receptors in conjunction with Cl^- influx. This is an unusual pathway as normally steroids bind to nuclear receptors while GABA receptors are membrane bound. These observations are interesting given the high concentrations of progesterone and other membrane-active steroids in the cumulus following ovulation. However, until the molecular mechanisms are fully characterized it is probably inappropriate to use follicular fluids on a routine basis for sperm stimulation or enhancement of motility or fertilizing ability.

Capacitation assays

Human sperm capacitation status can be monitored by a chlortetracycline fluorescence technique originally developed for mouse spermatozoa. The assay depends on the binding of chlortetracycline and Ca^{2+} to the sperm surface during the early stages of capacitation. Chlortetracycline forms a fluorescent compound with Ca^{2+} and has been used in several physiological systems (such as blood platelets) to monitor Ca^{2+} fluxes. As sperm capacitation continues fluorescence gradually disappears, presumably as a result of Ca^{2+} influx. The technique has the advantage that small aliquots of sperm populations can be removed and the chlortetracycline-fluorescence 'frozen' with glutaraldehyde for later evaluation. The assay probably cannot distinguish between physiologically normal and spontaneous acrosome reactions, and needs controls for sperm viability. Nevertheless, it is a valuable tool and has been used to demonstrate delayed capacitation patterns, and reduced inducibility of acrosome reactions in men with abnormal semen profiles such as teratozoospermy and polyzoospermy (Kholkute, Meherji & Puri, 1992).

Bronson's group in Stony Brook, NY, have examined the expression of glucoproteins such as vitronectin and fibronectin on the surface of human sperm following capacitation, and have shown a relationship between low levels of expression and impaired capacity to undergo a progesterone-induced acrosome reaction. This is consistent with observations that the egg surface has recognition sites to the ubiquitous RGD peptide (Arg–Gly–Asp) common to a number of extracellular matrix proteins. A variety of other factors could be used to study the onset of capacitation and the acrosome reaction. One such factor is fucoidin, which inhibits human sperm–zona binding and the zona-induced acrosome reaction. A very promising approach is that of Tesarik *et al.* (Tesarik *et al.*, 1991), who studied the appearance of D-mannose binding sites on the surface of sperm cultured under capacitating conditions. As described earlier, D-mannose on the zona pellucida may interact with D-mannosidase on the sperm surface during zone binding. Tesarik observed that spermatozoa from fertile men showed a characteristic pattern of D-mannose binding over the acrosomal region. This pattern differed in infertile men. The overall proportion of motile sperm showing D-mannose binding specifically over the acrosome was less than 10%, which is consistent with observations on the background rate of spontaneous capacitation and acrosome reactions. This interesting finding, if it can be repeated, may open the way to more specific tests based on the dynamics of sperm capacitation and exposure of zone-binding factors.

Zona pellucida binding and penetration

Sperm interaction with the zona pellucida consists of a number of separate components each one of these steps requiring active sperm involvement and is a potential source of fertilization failure (Storey, 1991). Besides enabling us to understand the molecular biology of sperm–egg interaction these separate components can also serve as very specific tests of sperm fertilizing ability *in vitro* (Oehninger, 1992). The components as presently understood are as follows:

1. Loose attachment
2. Firm binding and induction of the acrosome reaction
3. Penetration of the zona matrix.

Oocytes can be prepared in bulk from surgically removed ovaries or from post-mortem material using a combination of mechanical and enzymic dissection, screening the dissociated tissue through filters of decreasing size and finally by pipetting under a dissecting microscope. Care must be taken to select mature oocytes as there is evidence that immature oocytes show reduced sperm binding compared with mature. However, even 'mature' oocytes selected from large antral follicles may differ significantly in binding and sperm penetrating characteristics despite having very similar appearance. Clearly, 'zona maturation', along with nuclear and cytoplasmic maturation of the oocytes, is another factor to consider both in designing binding assays and in understanding how differences in IVF fertilization rates are caused by inherent oocyte factors. Alternatively, tests may use surplus oocytes from IVF programmes, and even unfertilized oocytes that have been exposed to spermatozoa (this may be subject to ethical approval and legal constraints). In the latter case care must be taken to distinguish any residual spermatozoa remaining from the IVF attempt from any that are used to assess zona binding or penetration. In addition some oocytes may be activated during the IVF attempt resulting in reduced sperm binding capacity. In all cases internal controls, both positive and negative, must be used to take account of variation between oocytes and between women. This is particularly important for comparisons between centres.

As the zona material is metabolically relatively inert, it is possible to bulk-store zonae either by freezing or by the much more convenient approach of salt-storing in buffered hyperosmolar solutions. Studies have shown very similar sperm binding characteristics between oocytes pickled in this way (1.5M $MgCl_2$ with 0.1% Polyvinulpyrrolidone in HEPES buffer) and those cryopreserved in 2M DMSO: also between salt-stored and fresh oocytes (Oehninger, 1992).

Two basic approaches have been used to assess and control sperm binding and penetration. In both cases, test spermatozoa are compared with those from a fertile donor. In competitive binding assays the sperm are labelled with different fluorochromes and then mixed in approximately equal numbers. This has a twofold disadvantage: first the fluorochrome used may itself affect sperm viability or binding capacity (or both); secondly large number of oocytes must be used to account for between-oocyte variability. The hemi-zona assay pioneered by the Jones Institute in Norfolk, Virginia uses oocytes that are bisected by a micro-manipulator. One half zone is inseminated with test spermatozoa and the other control from a fertile donor. In this way each oocyte serves as its own control, and fewer oocytes are need to attain similar levels of accuracy to the competitive assay (Oehninger, 1992). The hemi-zona assay has proved very useful in assessing inherent fertilizing ability, as well as the dynamics of capacitation, hyperactivation and the acrosome reaction (Burkman, 1990). Fertile sperm cultured under capacitating conditions for a hemi-zona assay showed a peak of hyperactive movement at about 2 hours followed by a slow decline over 5 hours. There was an increase in the numbers of spontaneously acrosome reacting spermatozoa coupled with a decrease in acrosome intact spermatozoa between and 3 and $3\frac{1}{2}$ hours. Sperm–zona binding increased rapidly between 30 minutes and 2 hours and after $3\frac{1}{2}$ hours there was a steady decline in the capacity of the sperm population to initiate firm zona binding.

Penetration of heterologous oocytes

A major breakthrough in functional tests for sperm fertilizing capacity came when Yanagimachi demonstrated that the zona-free golden hamster oocyte could support the penetration of 'foreign' spermatozoa. In hamsters the major block to polyspermy lies in the zona pellucida, while the oocyte surface is remarkably non-discriminatory even after penetration and egg activation. Work from Yanagimachi's group has demonstrated that, unlike any other known species, the hamster oocyte will permit the penetration of multiple foreign spermatozoa. The capacity to fuse with spermatozoa develops in the forming oocyte at about the time the zona pellucida and the egg microvilli appear. It declines after sperm penetration and is lost by the eight-cell embryo stage. The nature of the fusogenic factor is obscure, as it resists proteinase digestion. It may be a glycolipid or a cryptic membrane protein.

Since the original observation by Yanagimachi *et al.* in 1976, the zona-free hamster oocyte penetration test has developed as a fundamental labroatory element in the diagnosis and treatment of male infertility. It has been applied to predicting fertility, evaluating function after contraception or medical therapy to

alleviate infertility, and in assessing semen after cryopreservation. One very important clinical application of the test is in karyotyping human sperm as under careful control the penetrating spermatozoon will form a metaphase plate, and this end-point has been used to monitor possible genetic damage to sperm (Martin, 1989). The hamster egg has also been used to study the fertilizing ability of sperm from a wide range of animal species, from dolphins to birds.

In essence, the hamster egg test involves inducing superovulation in prepubertal (4–6 weeks old) female hamsters. Eggs are removed from the oviduct at a defined time after ovulation and treated with hyaluronidase to remove the cumulus. Trypsin digestion removes the zona pellucida. A standard concentration of motile spermatozoa is added to groups of eggs. Following a defined period of co-incubation (usually 3 h at 37 °C under humid 5% CO_2 in air) the eggs are removed, washed and examined for the presence of penetrating sperm heads associated with tails. The results are usually read as a penetration rate (percentage of eggs with decondensing sperm heads). However, as polyspermic penetration is common (particularly with fertile men) the penetration rate may be combined with information about the numbers of sperm heads per penetrated egg. The conditions need to be very carefully standardized with meticulous attention to media type and purity, water source and brand of tissue culture ware, as all these factors can affect the results.

While the hamster egg test has been widely applied, there are great problems in standardizing the methodology, and historically this caused unacceptable levels of false-negative results (i.e. fertile men showing failure to penetrate). Aitken (Aitken & Elton, 1984) showed that the interaction between sperm and eggs conforms to Poisson distribution theory, meaning that observed fertilization rates can be explained purely on the grounds of collision frequency between receptive oocytes and capacitated spermatozoa. This work led to a standard recommended protocol (World Health Organization, 1987), although much work still continues in attempts to reduce the variability of the test. The essential problem, which is of course common to homologous IVF, is that penetration requires collision between a capacitated spermatozoon that is ready to undergo the acrosome reaction and an oocyte. The motile sperm population in any culture consists of a heterogenous set of sperm, at different stages of capacitation, and a major subset may be incapable of completing the acrosome reaction. A variety of strategies have been advanced to maximize the number of reacting sperm and thus the penetration rate. The philosophy is that if we can induce *all* competent sperm to fertilize then we obtain the maximum amount of information about the population, thus enhancing the capacity of the test to discriminate between fertile and sub-fertile men. Strategies include pre-incubation in medium where Sr^{2+} replaces Ca^{2+}, incubation in high Ca^{2+} or hypertonic medium, and prolonged

incubation at low temperature (4 °C) in TEST-egg yolk buffer. Why the latter approach works is not clear: either the stress differentially kills incompetent spermatozoa or the lipid component of the medium enhances capacitation by enhancing cholesterol efflux from the sperm membrane or otherwise affecting lipid phase transitions. Aitken's group has shown that treatment with A23187 to induce the acrosome reaction results in more consistent hamster egg test results that are better predictors of fertility after artificial insemination or IVF. In addition, the increased penetration rates allow for more economical use of hamster oocytes.

Despite these advances, the hamster ovum test remains a very expensive and labour-intensive mode of studying infertility. For many clinics the cost is prohibitive, and in Australia it is banned in several states. There are thus pressing reasons to develop alternative tests of similar predictive value.

Penetration of homologous oocytes

The definitive test of sperm function is, of course, the ability to fertilize homologous oocytes and to initiate embryonic development. However, a single episode of fertilization failure does not necessarily mean that subsequent attempts will also fail. A number of IVF units have proposed using 'spare' oocytes from IVF attempts and it is indeed possible to freeze aged oocytes and use them subsequently (following removal of the zona pellucida) in a test of fertilizing capacity. This approach depends, of course, on ethical and legal authorisation. In Western Australia it would be illegal, as both the oocyte in the process of fertilization and the resulting embryo are protected in law, and an embryo can only be created with the intention of allowing it to develop normally (paradoxically, embryos once created can be 'allowed to succumb' but cannot be used for research).

Conclusions

No single test of sperm function yet exists that will successfully predict all aspects of sperm function. Fertilization failure *in vitro* is the area of assisted reproduction that causes most distress in practice. In some cases (general biochemistry, capacitation assays and homologous zona binding or penetration assays) the cost will vary widely according to the specific test being performed, and also the degree of access to material. Some tests may well be banned in different countries due to ethical or legal constraints.

What can we predict of the future for sperm testing? There are a number of interesting possibilities. It may prove possible to separate 'good' from 'bad' sperm subsets by their surface characteristics or swimming behaviour. Monoclonal

antibody-coated magnetic beads could separate acrosome-intact from acrosome reacted spermatozoa. Separation of sperm in different phases of capacitation may be possible using lectin-coated beads specific for different glycoproteins and their associated sugar moieties. Alternatively, we can start to look for the expression of exposure of specific membrane proteins as sperm capacitate. Molecular synthesis of pure human zona pellucida protein fractions such as ZP3 will give us an invaluable research tool and could serve to separate capacitated from non-capacitated sperm based on their ZP3 binding affinity. It is known that some aspects of sperm function including capacitation are modified by neurohormones and changes occur in the distribution of cholinergic binding sites during capacitation in the rabbit. If true for human spermatozoa, this could provide another route for detecting and separating capacitated from non-capacitated spermatozoa. Any of these approaches could also be used in flow cytometry. A major challenge in this approach is to develop staining and separation techniques that do not damage spermatozoa and which allow viable cells to be recovered quickly so that they can be used in assisted reproductive technology.

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References

Aitken RJ. Motility parameters and fertility. In: Gagnon C, ed. *Controls of Sperm Motility: Biological and Clinical Aspects*. Boca Raton: CRC Press, 1990: 285–302.

Aitken RJ, Bowie H, Buckingham D, Harkiss D, Richardson DW, West KM. Sperm penetration into a hyaluronic acid polymer as a means of monitoring functional competence. *J Androl* 1992*a*; **13**: 44–54.

Aitken RJ, Buckingham D, West K, Wu FC, Zikopoulos K, Richardson DW. Differential contribution of leucocytes and spermatozoa to the generation of reactive oxygen species in the ejaculates of oligozoospermic patients and fertile donors. *J Reprod Fert* 1992*b*; **94**: 451–62.

Aitken RJ, Elton RA. Significance of Poisson distribution theory in analysing the interaction between human spermatozoa and zona-free hamster oocytes. *J Reprod Fert* 1984; **72**: 311–21.

Boyers SP, Davis RO, Katz DF. Automated semen analysis. *Curr Probl Obstet Gynecol Fertil* 1989; **12**: 165–200.

Burkman LJ. Hyperactivated motility of human spermatozoa during *in vitro* capacitation and implications for fertility. In: Gagnon C, ed. *Controls of Sperm Motility: Biological and Clinical Aspects*. Boca Raton: CRC Press, 1990: 303–29.

Byrd W, Wolf DP. Acrosomal status in fresh and capacitated human ejaculated sperm. *Biol Reprod* 1986; **34**: 859–69.

Chan PJ, Tredway DR. Association of human sperm nuclear decondensation and *in vitro* penetration ability. *Andrologia* 1992; **24**: 77–81.

Cummins JM. Evolution of sperm form: levels of control and competition. In: Bavister BD, Cummins JM, Roldan ERS, eds. *Fertilization in Mammals*. Norwell, Massachusetts: Serono Symposia, USA, 1990: 51–64.

Cummins JM, Pember SA, Jequier AM, Yovich JL, Hartmann PE. A test of the human sperm acrosome reaction following ionophore challenge (ARIC): relationship to fertility and other seminal parameters. *J Androl* 1991; **12**: 98–103.

Davis RO. The promise and pitfalls of computer-aided sperm analysis. *Infert Reprod Med Clinics N America* 1992; **3**: 341–52.

Davis RO, Niswander PW, Katz DF. New measures of sperm motion. I. Adaptive smoothing and harmonic analysis. *J Androl* 1992; **13**: 139–52.

Eisenbach M, Ralt D. Precontact mammalian sperm–egg communication and role in fertilization. *Am J Physiol* 1992; **262**: C1095–101.

Ford WCL, Rees JM, McLaughlin EA, Goddard RJ, Hull MGR. The effect of A23187 concentration and exposure time on the outcome of the hamster egg penetration test. *Int J Androl* 1991; **14**: 127–39.

Hoskins D, Vijayaraghavan S. A new theory on the acquisition of sperm motility during epididymal transit. In: Gagnon C, ed. *Controls of Sperm Motility: Biological and Clinical Aspects*. Boca Raton: CRC Press, 1990: 53–62.

Huszar G, Vigue L, Morshedi M. Sperm creatine phosphokinase M-isoform ratios and fertilizing potential of men: a blinded study of 84 couples treated with *in vitro* fertilization. *Fert Steril* 1992; In Press.

Ivani KA, Seidel GE. At least half of capacitated, motile mouse sperm can fertilize zona-free mouse oocytes. *J Exp Zool* 1991; **412**: 406–12.

Jeyendran RR, Van der Ven HH, Perez-Pelaez M, Crabo BG, Zaneveld LJD. Development of an assay to assess the functional integrity of the human sperm membrane and its relationshiop to other semen characteristics. *J Reprod Fert* 1984; **70**: 219–28.

Johnson L, Varner DD. Effect of daily spermatozoan production but not age on transit time of spermatozoa through the human epididymis. *Biol Reprod* 1988; **39**: 812–17.

Katz DF, Andrew JB, Overstreet JW. Biological basis of *in vitro* tests of sperm function. *Prog Clin Biol Res* 1989; **302**: 95–103.

Katz DF, Overstreet JW, Hanson FW. A new quantitative test for sperm penetration into cervical mucus. *Fert Steril* 1980; **33**: 179–86.

Kessopoulou E, Tomlinson MJ, Barratt CLR, Bolton AE, Cooke ID. Origin of reactive oxygen species in human semen: spermatozoa or leucocytes? *J Reprod Fert* 1992; **94**: 451–70.

Kholkute SD, Meherji P, Puri CP. Capacitation and the acrosome reaction in sperm from men with various semen profiles monitored by a chlortetracycline fluorescence assay. *Int J Androl* 1992; **15**: 43–53.

Martin RH. Analysis of human sperm chromosome complements. In: Bavister B, Roldan E, Cummins J. eds. *Fertilization in Mammals*. Norwell, Massachusetts: Serono Symposia, USA, 1989: 365–72.

Morrell JM. Applications of flow cytometry to artificial insemination: a review. *Vet Rec* 1991; **129**: 375–8.

Mortimer D. Semen analysis and sperm washing techniques. In: Gagnon C, ed. *Controls of Sperm Motility: Biological and Clinical Aspects*. Baco Raton: CRC Press, 1990: 263–84.

Ng S-C. Micromanipulation: its relevance to human *in vitro* fertilization. *Fert Steril* 1990; **53**: 203–19.

Oehninger S. Diagnostic significance of sperm-zona pellucida interaction. *Reprod Med Reviews* 1992; **1**: 57–81.

Palermo G, Joris H, Derde M-P, Camus M, Devroey P van Steirteghem A. Sperm characteristics and outcome of human assisted fertilization by sub-zonal insemination and intracytoplasmic sperm injection. *Fertil Steril* 1993; **59**: 826–35.

Serres C, Feneux D, Jouannet P. Abnormal distribution of the periaxonemal structures in a human sperm flagellar dyskinesia. *Cell Motil Cytoskel* 1986; **6**: 68–76.

Stephens DT, Hoskins DD. Computerized quantitation of flagellar motion in mammalian sperm. In: Gagnon C, ed. *Controls of Sperm Motility: Biological and Clinical Aspects*. Boca Raton: CRC Press, 1990: 251–60.

Storey BT. Sperm capacitation and the acrosome reaction. In: Robaire B, ed. *The Male Germ Cell: Spermatogonium to Fertilization*. 637. New York: NY Academy of Sciences, 1991: 459–73.

Suarez SS, Katz DF, Owen DH, Andrew JB, Powell RL. Evidence for the function of hyperactivated motility in sperm. *Biol Reprod* 1991; **44**: 375–81.

Talbot P, Chacon R. A triple-stain technique for evaluating normal acrosome reactions in human sperm. *J Exp Zool* 1981; **215**: 201–8.

Tesarik J. Appropriate timing of the acrosome reaction is a major requirement for the fertilizing spermatozoon. *Human Reprod* 1989; **4**: 957–61.

Tesarik J, Mendoza C, Carreras A. Expression of D-mannose binding sites on human spermatozoa: comparison of fertile donors and infertile patients. *Fert Steril* 1991; **56**: 113–18.

Tombes RM, Shapiro BM, Metabolite channelling. A phosphorylcreatine shuttle to mediate high energy phosphate transport between sperm mitochondrion and tail. *Cell* 1985; **41**: 325–34.

Wassarman PM. Fertilization in the mouse. 1. The egg. In: Dunbar BS, O'Rand MG, eds. *A Comparative Overview of Mammalian Fertilization*. NY: Plenum Press, 1991: 151–65.

World Health Organization. WHO laboratory manual for the examination of human semen and semen – cervical mucus interaction. In: eds. *WHO Laboratory Manual for the Examination of Human Semen and Semen – Cervical Mucus Interaction*. Cambridge: Cambrdge University Press, 1987.

World Health Organization Task Force on the Prevention and Management of Infertility. Adenosine triphosphate in semen and other sperm characteristics: their relevance for fertility prediction in men with normal sperm concentration. *Fert Steril* 1992; **57**: 877–81.

Yanagimachi R. Mechanisms of fertilization in mammals. In: Mastroianni L, Biggers JD, eds. *Fertilization and Embryonic Development In Vitro*. New York: Plenum Press, 1981; 81–182.

Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD, eds. *The Physiology of Reproduction*. New York: Raven Press, 1988*a*: 135–85.

Yanagimachi R. Sperm-egg fusion. In: Duzgunes N, Bronner F, eds. *Current Topics in Membrane and Transport*. 32. NY: Academic Press, 1988*b*: 3–43.

Yanagimachi R, Yanagimachi H, Roger BJ. The use of zona-free animal ova as a test-system for the assessment of the fertilizing capacity of human spermatozoa. *Biol Reprod* 1976; **15**: 471–6.

Yudin AI, Gottlieb W, Meizel S. Ultrastructural studies of the early events of the human sperm acrosome reaction as initiated by human follicular fluid. *Gamete Res* 1988; **20**: 11–24.