

Fertility evaluation of frozen/thawed semen

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Abstract

In vitro semen analyses have been used for more than half a century to estimate the fertilizing potential of a semen sample. Unfortunately, none of the assays developed provide results that consistently correlate well with fertility. The reasons for this lack of consistency, due in part to the complexity of the spermatozoon itself, the collection of fertility data, and factors beyond control of the semen analyses themselves, are discussed. Different spermatozoal attributes that are necessary for a spermatozoon to fertilize an oocyte are presented and assays used to evaluate each attribute described. Although laboratory assay results do not correlate well with semen fertility, the importance of conducting laboratory assays on every semen sample used for artificial insemination or to attempt to determine causes for infertility, is discussed.

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1. Introduction

Since the advent of AI, researchers have sought laboratory assays that would accurately predict the fertilizing potential of a semen sample. This goal, however, has proven difficult to achieve. This difficulty arises from the complex nature of the problem, dealing with the complexities of the spermatozoon itself, with our ability to evaluate fertility, and with our ability to manage the females to be inseminated.

Part of the complexity dealing with the spermatozoa, comes from the fact that each spermatozoon is a multicompartamental cell that must possess many different attributes to be able to fertilize an oocyte. Each spermatozoon must possess: (1) motility; (2) active

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mitochondria to supply the energy necessary for motility; (3) intact acrosomal membranes that are capable of undergoing capacitation changes thereby permitting the acrosome reaction to occur, but only at the correct moment; (4) receptors that permit the cell to bind to the zona pellucida and to the oolemma; (5) plasma membranes that permit fusion with the oolemma and (6) a nucleus that is capable of proper decondensation, nuclear reorganization, and genetic performance to maintain zygotic and embryonic development (just to name a few). Over the past 75 year, researchers developed laboratory assays for many of the attributes spermatozoa require to fertilize an oocyte [1–4], but there are likely many sperm attributes necessary to fertilize an oocyte that are unknown. There may also be problems inherent in a specific assay that may limit the usefulness of the data obtained using that assay [2,5]. In addition, because each spermatozoon requires many attributes to fertilize an oocyte, an assay measuring only a single attribute will fail to detect spermatozoa defective in a different attribute, and therefore, overestimate the number of fertile spermatozoa in a semen sample (Fig. 1).

Unfortunately, because these assays evaluate a population mean for each attribute measured, combining results from two or three different assays on the same sperm population does little to determine the ‘true number of fertile sperm’ in a population. To achieve this, we would need to assess all the attributes necessary for fertilization simultaneously on each individual spermatozoon, as depicted in Fig. 1.

Problems also exist in defining and measuring fertility. Fertility can be described as the percentage of females from which embryos are flushed after insemination, the percentage of females that are pregnant a defined number of days after a single insemination or multiple inseminations during a single heat cycle or several heat cycles, or the percentage of females that deliver a live offspring after insemination (again single or multiple inseminations over one or many heat cycles) [1,5]. Each of these is a very different response and each reflects a different aspect of maternal input that is not likely to be detected in a semen assay [5]. In addition, for fertility data to be useful, it must accurately reflect the true fertilizing potential of the semen used [5]. Since fertility is a binomial variable, relatively large numbers of females must be inseminated in order to achieve precise, accurate fertility data. If only 10 females are inseminated, for example, the variation for the fertility data will be about $\pm 25\%$ [5,7]. Therefore, if 5 of 10 females

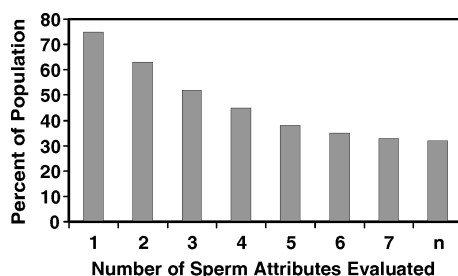


Fig. 1. Hypothetical sub-populations of spermatozoa in a semen sample considered to be “fertile” when one attribute (1), two attributes (2), or greater numbers of attributes are measured simultaneously (adapted from Amann and Hammerstedt [6]).

become pregnant, true fertility of that semen sample could range from 25 to 75% [5,7]. In order to determine a true fertility percentage with a variation of approximately $\pm 10\%$, nearly 75 females per treatment must be inseminated, and at least 100 females must be inseminated to reduce the variation to $\pm 7\%$ [5,7]. Very few fertility studies are conducted that inseminate sufficient numbers of females to produce reliable fertility data to correlate semen analysis data with [5].

There are also few studies with sufficiently large numbers of males that have a wide variation in fertility (possibly from 5 to 85%) to determine accurate correlations between laboratory assay results and fertility. Most studies that utilize males with fertility ranges sufficiently wide to detect possible fertility differences, inseminate too few females per male to produce reliable fertility data. Erstwhile, studies utilizing sufficiently large numbers of females to produce reliable fertility data utilize males with insufficient variation in fertility to determine accurate correlations between fertility and laboratory semen analyses [5].

Finally, fertility is also dependent upon the number of spermatozoa inseminated [8–10], the age of the females being inseminated [11,12], and timing of insemination to ovulation [5,12]. These variables are not detected in semen analysis.

Despite difficulties correlating laboratory data with fertility, laboratory evaluation of semen quality remains important. Although laboratory semen analyses may not predict actual fertilizing potential of a semen sample, the analysis may predict samples of low fertility and allow exclusion of those samples from an artificial insemination program [1,3,5,7].

2. Types of laboratory semen analyses

Laboratory semen assays can be classified in several ways. One major distinction is between direct and indirect assays. Direct assays evaluate actual cells individually, while indirect assays measure a component of the entire sample, such as the amount of an enzyme released from the entire sperm sub-sample [2]. Although both types of assays can delineate important attributes of spermatozoa, this review will focus mainly on direct assays. Within the category of direct assays, sub-categories of assays can be distinguished in which semen is evaluated using manual or automated techniques.

2.1. Manual/visual sperm analyses

Initially, semen assays were conducted by placing a sub-sample from the semen sample on a microscope slide and visually evaluating the sub-sample. These types of assays using fresh or fixed, unstained or stained sub-samples, remain a mainstay of the assays conducted by most laboratories.

2.1.1. Unstained spermatozoa

Fresh unstained spermatozoa are commonly examined microscopically, to estimate the percentage of motile sperm in a semen sample. Such estimations can include both the percentages of total motile cells, as well as progressively motile cells. In addition, sub-

samples can be fixed with formaldehyde or glutaraldehyde, and the morphology of the cells evaluated using phase contrast or differential interference contrast (DIC) microscopy (see review [3]).

2.1.2. Stained spermatozoa

These analyses usually require a spermatozoal sub-sample to be stained to enhance visualizing the sperm (IVF or zona binding assays) or differences in organelle structure (dried spermatozoal smears on a microscope slide) that can be evaluated visually using microscopy [3,13–17].

2.2. Automated spermatozoal analyses

Manual analyses can suffer from human bias [2] as well as being time consuming, either in the sample preparation or analysis itself, resulting in relatively few (usually ≤ 200) spermatozoa being evaluated. Therefore, in recent years, techniques have been developed to assess the same sperm attributes originally evaluated manually using automated technologies.

2.2.1. Computer assisted sperm analysis (CASA)

CASA systems permit the evaluation of sperm motility in a relatively non-biased manner. These systems also permit the velocities of spermatozoa to be determined. In addition, some CASA systems are equipped with capabilities for evaluating the morphology of spermatozoa.

2.2.2. Flow cytometry

Flow cytometry is a powerful tool for evaluating cells. It utilizes technologies that force individual cells into a confined stream of fluid that passes through a laser beam. If the cells have been previously stained with fluorescent dyes, dyes associated with the cells will fluoresce, and the fluorescent light from each individual cell can be detected by photomultiplier tubes contained within the machine. The power of this technology is that approximately 50,000 cells can be counted in a minute, several different dyes can be added to cells at the same time so that cells can be evaluated for different attributes simultaneously, the staining techniques are all very simple and rapid, fixed or live cells can be evaluated and fluorescent probes are currently available to evaluate nearly any cell attribute one would wish to measure.

3. Analyses for attributes of cryopreserved spermatozoa

3.1. Sperm morphology

Common classification systems for the morphology of spermatozoa from different species have been reported [3]. However, classification categories are different for the various species and the adoption of a uniform system within each species is needed. Spermatozoal abnormalities can be divided into primary and secondary abnormalities or in

some classification systems major and minor abnormalities. Primary abnormalities are believed to arise in the testis during spermatogenesis, while secondary abnormalities are believed to occur after the sperm has left the testis. Classification systems utilizing major and minor categories put less emphasis on where defects arise, but emphasize their overall effect on subsequent fertility. Therefore, major defects are those associated with impaired fertilizing potential, while minor defects have little effect on fertility [18], and can usually be compensated for by adding more spermatozoa in the inseminate [10].

It is unlikely that cryopreservation induces major changes in the morphology of spermatozoa. However, poor handling techniques or sub-optimal cooling and freezing conditions may induce irreversible changes such as acrosomal damage or reflex of the sperm tail [3].

3.1.1. Visual assessment

Visual microscopic examination of fixed cells, unstained wet samples or stained dried samples, has been conducted for many years to evaluate the percentages of morphologically normal cells in a semen sample. Reports indicate that as the percentage of morphologically abnormal cells increases in a semen sample, the fertility of the samples decrease [10,13,17]. This is particularly true for samples containing high numbers of spermatozoa with abnormal heads [10], nuclear vacuoles [19,20], and abnormal acrosomes [13].

3.1.2. Morphometry

Although a subjective assessment of spermatozoal head shape can be conducted visually, objective methods for measuring sperm nuclear shape have been developed using digital images of sperm. Automated sperm head morphometry analysis systems determine head length, width, and area and calculate permutations of these data such as width/length and sperm perimeter, that have been used to categorize stallions into fertile and sub-fertile groupings [21–24]. Digital spermatozoal images can also be analyzed using Fourier functions to determine Fourier harmonic amplitudes of individual spermatozoa [25]. A high relationship ($r = 0.89$, $P < 0.05$) has been reported between sperm nuclear shape and the fertility of bull spermatozoa using this technique [26].

3.2. Sperm plasma membrane integrity

The integrity of the sperm plasma membrane is often synonymous with spermatozoal viability, and most ‘viability assays’ actually assess whether or not the cell plasma membrane is intact or not. In order to fertilize an oocyte, a sperm must have an intact and competent plasma membrane. However, plasma membrane destabilization during the freeze/thaw cycle causes intracellular ice formation [27], a major cause of cell death during cryopreservation [28]. This membrane destabilization and/or disruption occur when temperature is decreased and the membrane undergoes a phase transition from the fluid-phase to the gel-phase. One of the consequences of membrane disruption is the loss of intracellular components, such as metabolic enzymes and ATP, which ultimately leads to cell death.

3.2.1. Visual assessment

The integrity of the sperm plasma membrane can be assessed visually using light or fluorescent microscopy. The plasma membrane of the spermatozoa, however, is not a homogenous membrane but is actually compartmentalized into several domains and different assays often assess only one domain. For example, the hypo-osmotic swelling test, in which spermatozoa are incubated in hypo-osmotic media (usually from 50 to 150 mOsm, depending upon species) enables assessment of the plasma membrane covering the principle piece [1,29,30]. For this assay, sperm are incubated in a hypo-osmotic medium, and then assayed, using light microscopy. If the plasma membrane over the principle piece is intact, the membrane will swell causing the tail to coil, while spermatozoa with damaged principle piece membranes will not swell.

The integrity of membranes covering the sperm head is assessed after staining the cells. Eosin–nigrosin or eosin aniline blue stains [3] are commonly used for dried sperm smears, while the combination of carboxyfluorescein diacetate (CFDA) with propidium iodide (PI), or SYBR-14 with PI are commonly used to stain wet smears, but must be assessed using fluorescent microscopy. PI is membrane-impermeant red fluorescent molecule that enters the nucleus of a cell in which the plasma membrane is damaged. CFDA is a membrane-permeant colorless substrate that is rapidly converted by intracellular esterases into a membrane-impermeant green fluorescent derivative [1,31]. Used in combination, cells with damaged membranes fluoresce red as PI enters the cell, and intracellular esterases leak from the cell, therefore, CFDA is not converted its green derivative. Cells with intact membranes fluoresce green as the membranes prohibit PI entry and the retained esterases convert CFDA into a green derivative.

In contrast, SYBR-14 is a membrane-permeant green fluorescent probe that binds to the DNA in the nucleus of all cells, both membrane intact and membrane compromised. When used in combination with PI, cells with intact plasma membranes only stain with SYBR-14 and fluoresce green, while cells with damaged plasma membranes stain with both SYBR-14 and PI and fluoresce red, or red-orange as the fluorescence of the PI is brighter than that of the SYBR-14 [32].

3.2.2. Fluorometry

Fluorometry is an indirect method of assessing the percentage of membrane-intact cells in a semen sample [33]. In this technique, a semen sample is treated with a membrane impermeant fluorescent probe, such as ethidium bromide, diamidino-phenylindole dihydrochloride (DAPI), or Hoechst 33258, that binds to the DNA of cells with damaged membranes causing them to fluoresce. The sample is then assayed with a fluorometer that provides fluorescence intensity of only the ‘dead’ cells in the sample. The sample is then treated with digitonin, which permeabilizes all the cells and the sample assayed a second time. The ratio of the fluorescence intensity of the first analysis compared to the fluorescence intensity of the second analysis provides the percentage of dead cells in the population. Additionally, spectropic assays for released enzymes have also been used to assess the percentages of viable cells in a sperm population [2]. The advantages of these assays compared to visual analysis are the rapidity of the assays and the ability to assess a large number of cells.

3.2.3. Flow cytometry

Using the same fluorescent probe combinations (CFDA/PI or SYBR-14/PI), flow cytometry can directly determine the percentage of membrane-intact cells in a sperm population [15,31,32].

3.3. Acrosomal membrane integrity

A spermatozoon must maintain an intact acrosome up to the time it binds to the zona pellucida of the oocyte and undergoes the acrosome reaction, which releases the acrosomal enzymes permitting the sperm to digest a hole through the zona pellucida, thereby allowing the spermatozoa access to the oolemma [34]. Methods for visually evaluating acrosomal status generally require the spermatozoa to be stained and have been extensively reviewed [3,14]. Fluorometry [35] and flow cytometry [4,15,36,37] can also be used to evaluate the acrosomal status of large populations of sperm.

3.4. Mitochondrial function

The mitochondria provide a majority of the ATP necessary for total spermatozoal metabolism, including that necessary for sperm motility. This review will address evaluation of sperm motility to assess mitochondrial function.

3.4.1. Sperm motility

The visual estimation of the percentage of motile spermatozoa in a semen sample is likely the most common semen analysis conducted in the laboratory. More recently, many laboratories evaluate sperm motion parameters using CASA or time-lapse photography using conventional or digital cameras to reduce human bias and increase the number of motion parameters (sperm velocity) that can be determined. Although a very important assay, motility analyses evaluate only one spermatozoal attribute necessary to fertilize an oocyte, and therefore, are not consistently highly correlated with fertility [2].

3.4.2. Direct mitochondrial evaluation

Several fluorescent probes have been used to evaluate sperm mitochondrial function. Mitochondrial probes are actively transported into actively respiring mitochondria, therefore, the more active the mitochondrial respiration, the more probe is accumulated. Rhodamine 123 was initially used to evaluate sperm mitochondrial function [15,38], but can only differentiate between respiring and non-respiring mitochondria. More recently, JC-1 has been used to assess spermatozoal mitochondria function [39,40]. At low concentrations, JC-1 remains in the monomeric state and fluoresces green. However, at high concentrations, JC-1 forms aggregates that fluoresce orange. Therefore, JC-1 has not only the ability to distinguish functional from non-functional mitochondrial, but permits different levels of mitochondrial function to be determined by intensity of mitochondrial ‘orangeness’. In support of sperm motility being a measure of mitochondrial function, the percentages of sperm with functioning mitochondria is highly correlated to sperm motility, regardless of whether rhodamine 123 [38,41] or JC-1 [39,40] is used to evaluate mitochondrial function.

3.5. Sperm capacitation

In order to fertilize an oocyte, a spermatozoon must first become capacitated and bind to the zona pellucida of the oocyte. Capacitation is a process in which many changes occur within the spermatozoon including but not limited to; cholesterol efflux from the plasma membrane [42,43], increases in intracellular calcium, bicarbonate, potassium, protein phosphorylation, and a decrease in intracellular pH [44–46]. In the best case scenario, a spermatozoon reaches the site of fertilization and completes capacitation at the time the oocyte is present. However, cryopreservation results in a loss of lipids from the sperm membranes [47] and a rearrangement of lipids and proteins within the membrane [48], which results in a “precapacitated” spermatozoa, resulting in a reduced fertilizing lifespan for the spermatozoon [49,50]. Therefore, assays to evaluate sperm capacitation can be used to evaluate the normalcy of spermatozoa after freezing and thawing, as well as to monitor techniques designed to induce sperm capacitation for assisted reproductive technologies.

Since there are many aspects of sperm capacitation many assays have been developed which monitor one or more parts of the capacitation process. Changes in membrane fluidity can be measured, using the probe merocyanin 540, as capacitation occurs [51]. Similarly, changes in intracellular calcium levels using chlortetracycline [14], Indo-1 AM [36] or fluo-3 AM [52], changes in protein phosphorylation [46,53] or the ability of sperm to undergo an acrosome reaction when challenged with various acrosome reaction-inducing compounds [35,54] can be assessed as sperm undergo capacitation.

3.6. Ability of sperm to bind to and fertilize oocytes

Assays that evaluate the ability of spermatozoa to bind to the zona pellucida and/or penetrate/fertilize oocytes *in vitro*, have also been developed. These assays evaluate the effectiveness (concentration and capacity) of sperm receptors to bind to the oocyte, the oolemma and initiate fertilization. Most of these assays require special equipment, are time consuming to perform, and are expensive to conduct.

3.6.1. Zona pellucida binding assay

In vitro assays in which the number of spermatozoa that bind to the zona pellucida (either whole or pieces of the zona pellucida) after incubation for a set period of time have been developed for spermatozoa from humans [55], bulls [56], boars [57], stallions [16] and other species. Part of the difficulty in conducting these assays is obtaining sufficient numbers of zona pellucidae. For this reason, partially dissected zona pellucidae are sometimes used. Molecular similarity between the zona pellucida and the perivitelline membrane of the hen's egg permit spermatozoa from many species [58,59], including stallion spermatozoa (Graham, unpublished) to bind to the perivitelline membrane. Setting up such an assay is simple and hen perivitelline membrane material is plentiful.

3.6.2. Oocyte penetration assay

The zona-free hamster oocyte penetration test was developed by Yanagimachi [60] to assess the fertilizing capacity of human spermatozoa [61]. Since then, this assay has been

adapted for spermatozoa from many species. More recently, zona-free bovine oocytes have also been used for bovine and stallion sperm penetration [52]. Since the zona pellucida is removed from the oocytes, this assay can evaluate the capacitation/acrosome status of the spermatozoa, as only sperm that have already undergone capacitation and an acrosome reaction can penetrate the oocyte [60–62]. This assay, however, does not evaluate the ability of spermatozoa to bind to and penetrate the zona pellucida, a formidable barrier to spermatozoa reaching the oocyte.

3.6.3. *In vitro fertilization assay*

Assays in which the ability of spermatozoa to fertilize homologous oocytes *in vitro*, have been developed for many domestic species, although techniques using equine gametes have not been repeatable [3]. In addition, culturing the produced zygotes for periods of time afterwards can also provide information on the genetic normalcy of spermatozoa [63]. Caution must be used in interpreting data collected as *in vitro* matured oocytes act differently than *in vivo* matured oocytes, both in their ability to become fertilized and in their subsequent development after fertilization has taken place.

3.7. *Sperm chromosomal and genetic integrity*

Not only does a spermatozoon have to get to the site of the oocyte, undergo timely capacitation, bind to the zona pellucida, undergo an acrosome reaction and fuse with the oolemma, it must also carry a nucleus that can properly decondense within the oocyte, fuse with the female pronucleus and possess genes, whose products facilitate normal embryonic development. Assays are currently being developed to evaluate these properties of spermatozoa.

3.7.1. *In vitro development*

Eid et al. [63] reported that bull spermatozoa from both high-fertility and low-fertility animals exhibited similar *in vitro* fertilization rates, but that the embryos produced by sperm from low-fertility bulls developed more slowly than embryos produced by sperm from high-fertility bulls. The authors hypothesize that higher numbers of sperm from low-fertility bulls possess genetic abnormalities, which delay the onset and rate of embryonic development [63,64].

3.7.2. *Sperm chromatin structure assay*

Nuclear abnormalities that affect gross chromosome structure may be detected in the overall shape of the sperm nucleus, while abnormalities in chromatin structure can be assessed by the susceptibility of chromatin to denature under certain conditions. Evenson et al. [65] developed the ‘sperm chromatin structure assay’ (SCSA), in which the level of denatured chromatin in sperm was assessed using the fluorescent probe acridine orange after sperm were incubated in denaturing conditions. Under these conditions, bull, mouse, and human spermatozoa from males with high fertility exhibited less denaturation than sperm from low-fertility males [65]. Similar results have been reported for boars [66], bulls [67], and stallions [68,69].

3.8. Simultaneous analysis of multiple spermatozoal attributes

Several laboratory assays have been developed that evaluate several spermatozoal attributes simultaneously on the same spermatozoon [14,15,37–39]. Such assays increase the likelihood of more accurately identifying sub-populations of fertile sperm in a sample and are more powerful than conducting single assessments of the same sperm attributes on different sub-samples of the semen sample, which may only still measure two or three attributes.

4. Correlating laboratory results with semen fertility

Correlations between laboratory results and fertility are inconsistent between studies [2,6,7]. Part of the reason for this is that sperm must possess many attributes to fertilize an oocyte, not merely one or two or even three attributes. Secondly, for most attributes, there is not a continuum from a minimum to a maximum that affects fertility, but instead a spermatozoa must possess a sufficient amount of each attribute to fertilize an oocyte [6]. Therefore, when evaluating a specific sperm attribute, we can postulate which samples are likely to have poor fertilizing capacity, but are unable to determine if a particular sample will be fertile. In addition, as mentioned previously, there are many variables (management of females to be inseminated, age of females to be inseminated, competence of inseminator, etc.) that have a marked effect on fertility outcome, but have nothing to do with semen itself [5]. Finally, our ability to determine ‘fertility’ accurately is dependent upon having sufficient numbers of inseminations from a particular semen sample [5]. Taken together, it is unlikely that any laboratory will consistently have laboratory assay results that correlate well with fertility.

5. Conclusions

Some of the basic laboratory assays for analyzing spermatozoa have been described as well as the spermatozoal attributes each assesses. Relatively few of these assays are conducted on every semen sample, and it is unreasonable that many of these assays should be conducted on every semen sample, due to time and expense. However, these assays might be performed on selected semen samples from males that are exhibiting low fertility to determine more precisely what spermatozoal attributes are compromised. Steps may then be taken to attempt to correct the deficiency, if possible. Unfortunately, it is very unlikely that results from any single laboratory assay will effectively estimate the fertilizing potential of a semen sample. However, laboratory assays are important, as they can help to eliminate poor samples from being used for artificial insemination and to determine what spermatozoal defects are present in samples with poor fertility.

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