Advances in canine semen evaluation techniques

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Abstract

Veterinarians are frequently asked to evaluate dog semen for a variety of reasons, including but not limited to: breeding soundness examination, shipment of fresh, cooled semen, cryopreservation; after a conception failure; after an illness suspect to affect fertility; or after testicular neoplasia or prostatic disease is diagnosed. Ultimately, the goal of any semen evaluation is to predict how likely it is for a male to be successful when used in a breeding situation. Standard semen evaluation in the dog includes determination of semen volume, spermatozoal motility (both total and progressive), velocity, concentration, total spermatozoa/ejaculate, and morphology. Semen morphology during a typical breeding soundness examination is typically performed using one of two stains: Wright-Giemsa or eosin-nigrosin. In the dog, standards for normal semen parameters include a semen concentration of > 10 million spermatozoa/kg bodyweight; >70% progressively motile sperm, and >70% morphologically normal spermatozoa. Many times, semen quality will exceed these parameters yet fertility of the dog may still be suboptimal. If infection and prostatic disease can be ruled out as causes of the subfertility, the clinician is left with attempting to further evaluate the ejaculate to determine the cause of the infertility. Evaluation of the sperm's function is the next logical step.

Male factor infertility is said to account for up to 50% of the failed pregnancy attempts in humans.[1] No estimates have been made for the canine, but clinical experience would tell us that male factor infertility accounts for a significant portion of either non-pregnancy, early embryonic death, or small litter size. When a standard semen evaluation fails to elicit a clear diagnosis of infertility, additional testing of the ejaculate is desirable. This testing may include additional bright field spermatozoal staining techniques which differentiate different parts of the

sperm cell; alternate microscopic evaluation of sperm morphology using differential interference contrast or phase contrast morphology; electron microscopy (EM), both scanning and transmission; acrosomal testing; hypo-osmotic swelling testing (HOST); sperm chromatin structure analysis (SCSA); computer assisted spermatozoal analysis (CASA/ASMA); fluorescent antibody staining techniques using flow cytometric analysis; anti-sperm antibody (ASA) testing; assays for reactive oxygen species (ROS); chromosomal studies; and sperm function testing including zona binding assays, sperm penetration assays.

While there is some information on the canine in this regard, much of the information presented will be from human, bovine, and other domestic species research, where this topic has been far more extensively evaluated. The data from other species can be extrapolated to the canine although more research is needed to determine if its use is appropriate in the dog as a predictor of fertility. This paper describes the use of these additional diagnostic methods of spermatozoal analysis for infertility assessment. CASA will only be covered briefly as another paper in this symposium is dedicated to its use.

Keywords: Canine, semen evaluation, male infertility

Introduction

In the human andrology laboratory setting strict criteria have been developed and accepted by the World Health Organization (WHO) for the evaluation of sperm morphology.[1-8] A normal human sperm has a specified size and shape, with a smooth outline, an acrosome that comprises between 40 and 70% of the sperm head, has no neck, midpiece or tail defects, and has no droplets more than ½ the size of the sperm' s head. Use of these strict criteria makes morphologic examination of semen more uniform and allows for easier comparison of research studies. Thus far, no such criteria have been adapted to the domestic species of animals, making semen evaluation less uniform and comparison of infertility studies and application of treatments more difficult.

Bright field microscopy

Bright field microscopy is one of the simplest forms of semen evaluation since every veterinary clinic possesses the equipment to perform the evaluation. There are many different stains which are available for morphologic assessment of spermatozoa. Commonly used stains include eosin-nigrosin (EN), modified Wright-Giemsa, Feulgen, India ink, and Spermac. Each stain has advantages and disadvantages and will be discussed individually.

EN stain has been a conventional stain used for semen morphology in domestic and non-domestic animals for many years.[8,9] Slides stained with EN have a dark background and a white or pink staining spermatozoon. The equatorial ridge can be clearly seen and some defects in the acrosomal cap and sperm head may be identified including knobbed or ruffled acrosomes, diadem defects, and nuclear vacuoles. The midpiece can be differentiated from the tail-piece or flagellum based on the change in thickness between the two structures. Midpiece defects such as thickening, roughening, proximal and distal cytoplasmic droplets, distal midpiece reflexes, coiling and bending may be determined. Tail defects including bending and coiling as well as duplicity may be diagnosed. EN is also a vital stain. Sperm that have intact membranes and normal chromatin will not allow eosin to be drawn into the cell and so stain white, while sperm with damaged membranes and damaged chromatin will stain pink due to eosin uptake. EN staining overestimates the number of intact membranes when compared to fluorescent assays (see flow cytometry section).[10] Use of live:dead information requires that proper staining procedure is used including prompt staining after collection, prevention of cold shock to the sperm, allowing adequate incubation time before smearing the slide, and rapid drying to prevent cracking or cell death. Round cells cannot be differentiated with this stain. The stain is hypotonic and this may result in stain artifact, such as coiled tails, if they are not dried properly. Cracks in the stain may also be noted if the smear is too thick.

Modified Wright-Giemsa stain (Diff-Quik®; Siemens Healthcare Diagnostics, Deerfield, IL, USA) is a simple, inexpensive, and rapid method of staining sperm.[9] Alterations in head

staining may correlate with DNA defects. The sperm head stains deeply basophilic from the equatorial region downward and more lightly basophilic in the area of the acrosomal cap. The midpiece and tail stain eosinophilic. Details of the acrosome are not visible with this stain, so only basic alterations in head size and shape are detectable. Midpiece and tail defects that can be visualized include cytoplasmic droplets, distal midpiece reflexes, bent and coiled midpieces and tails. This stain has the added benefit of allowing differentiation of round cells in the ejaculate, making identification of WBC vs. germ cells possible.

Feulgen staining is a time consuming procedure that allows for better visualization of sperm nuclear and head abnormalities.[9] The DNA in the sperm head stains magenta allowing good nuclear detail and making defects of the nucleus (vacuoles, diadem defects, etc) clearly visible. The acrosome, midpiece and tail do not stain. The stain must be made fresh daily or changes in the pH will affect the intensity of the stain. Slides may be evaluated with either bright field or phase contrast microscopy. The use of phase contrast microscopy results in greater color contrast. Feulgen staining identified more abnormalities of DNA pattern and head shape in the bull sperm nucleus than standard EN stain.[11]

India ink is a one-step stain that is simple and inexpensive to use. It provides a black background and a white or clear sperm outline. It does not allow for good visualization of the acrosome or equatorial region. It does however allow for differentiation of alterations in head size and shape, major defects of the midpiece including cytoplasmic droplets, distal midpiece reflexes, bent or coiled midpieces, and bent or coiled tails.

Spermac® (Conception Technologies, San Diego, CA, USA) is expensive and moderately time-consuming but is technically a simple staining technique.[12] It is important that thin smears of semen are made and are air dried for no more than 10 minutes prior to fixing. Once fixed, the remaining steps in the staining procedure may be delayed indefinitely. The nucleus of the cell stains dark red while the acrosome stains light green, and the midpiece and tail stain dark green. This is an excellent stain for acrosomal evaluation and also allows for

good midpiece and tail piece evaluation. Assessment of the nucleus is not as accurate, but head size and shape can be evaluated. Round cells can be visualized but they cannot be differentiated.

Papanicolaou stain is commonly used in human andrology labs, but is not commonly used in veterinary applications due to the complexity of the procedure.[8,13]

Phase contrast microscopy is a form of microscopy that allows small phase shifts of light through a transparent cell which are converted into contrast and amplitude changes in the image.[14] Two light rays are focused exactly inside the opening of the condenser annular ring. The two rays are refracted so that they exit the condenser in parallel. The light is minimally refracted on passing through the specimen and it travels in parallel into the objective where it enters the back focal plane of the objective. A phase plate is positioned in the back focal plane to line up with the condenser annulus.

Differential interference contrast (DIC) microscopy is a system whereby a polarized light source enters a prism and is diverted into two beams at 90 degree angles to each other.[14] These rays pass through a condenser and then the sample. The beams enter and pass through the sample about 0.2μm apart from each other. Since the beams pass through different parts of the cell they follow different optical paths. The beams then enter the objective lens of the microscope and passes through a second prism which recombines the rays into one polarized beam. This recombination leads to interference (since they are on different optical paths) which either brightens or darkens the image. The resultant image appears to be three-dimensional. DIC microscopy provides excellent resolution and clarity of cellular structure with minimal artifacts. It does require that the sperm sample is in a media of similar refractive index to the cells themselves which means that evaluation of semen in skim milk or yolk extenders is difficult.

No staining of the cell is required for either phase or DIC microscopy, but for morphology assessment, the sperm are generally fixed in formol buffered saline and viewed at high power

(40 – 100x). Both of these forms of microscopy provide for more detailed visualization of all parts of the sperm cell including the acrosome, nucleus, midpiece and tail piece than bright field microscopy alone.

Sperm morphologic assessment plays an integral role in predicting success with IUI, IVF and ART techniques.[3-8] Determination of teratozoospermia prior to attempting advanced insemination techniques will help with management choices. For example it has been shown in many human studies that by increasing sperm concentration at the time of insemination, patients with teratozoospermia will have a greater success rate with IUI and IVF procedures.[3-5] In humans it has also been repeatedly demonstrated that once the number of normal sperm drops below 14% infertility is a consistent result; with individuals with 0-4% normal forms have the lowest success rates (45%), 5 – 14% normal forms have moderate success rates (75%), while > 14% normal forms have good success rates (85%) when advanced reproductive techniques like IVF and ICSI are applied.[3-6]

Acrosome stains

Giemsa stain makes the acrosome appear dark purple.[9] It provides good detail of the acrosome but needs to be made fresh for each use and does not allow for evaluation of the sperm nucleus, midpiece or tail.

A sperm triple stain of Trypan blue stains the spermatozoa blue, Bismarck brown stained the post-acrosome region light brown and rose bengal stained the acrosome light red.[15] Sperm can be differentiated into four groups with this stain: dead sperm with fully or partially inactivated acrosomes, dead spermatozoa with missing or degenerated acrosomes, live spermatozoa with reacted acrosomes, and live spermatozoa with active or normal acrosomes. This staining technique has been used for humans, mouse, bull, horse, goat and boar semen.

A one step stain including fast green FCF, rose bengal and ethyl alcohol has been used to stain the acrosome of cat spermatozoa.[16] This stain allows differentiation of acrosome intact,

acrosome reacted or damaged sperm, and acrosome non-intact sperm. The slide is examined using bright field microscopy at 1000x.

Coomassie blue stain has been used to assess acrosomal integrity in bulls, boars, and stallions.[17,18] This staining procedure is relatively simple and results in intense blue staining of intact acrosome and lack of stain uptake in acrosome reacted sperm. The results of Coomassie blue staining correlate well with fluorescent staining (see below), DIC and bright field microscopy techniques following incubation with calcium ionophore to induce the acrosome reaction.[17]

Fluorescent stains, like acridine orange, can be used to evaluate sperm that are extenders in opaque extender, like skim milk.[9] Acrosomal integrity can be evaluated using fluorescent microscopy, phase-contrast or DIC microscopy.

Acrosome staining can be performed using bis-benzimide dye Hoescht 33 258 and a FITC-pisum sativum agglutinin (FITC-PSA) after induction of the acrosome reaction by calcium ionophore.[19] Sperm are incubated in TALP media and then calcium ionophore A23 187 is added to induce the acrosome reaction. Then the sperm suspension is permealized in methanol and incubated with a lectin in order to bind the FITC-PSA probe. The Hoescht 33 258 is then used to stain the sperm and fluorescent microscopy is used to differentiate acrosome reacted versus non-acrosome reacted sperm. It is a simple, quick technique to assess acrosome status, but it does not allow for morphologic assessment of the cell itself. Samples in egg yolk based extenders do not hinder using this technique.

Trypan blue or Congo red stain can be precipitated by neutral red and then stained with Giemsa to stain bull, boar and rabbit sperm, but not stallion sperm. This is a simple and reliable staining procedure that results in the stained sperm being classified as live or dead with intact acrosomes, loose or damaged acrosomes, detached acrosome and detached acrosome with no post acrosomal ring.[20]

Hypo-osmotic swelling test

This test is based on the concept that the normal sperm tail membrane will allow fluid to pass into the cell freely under hypo-osmotic conditions. As the fluid flows into the cell, the tail swells. Membrane integrity is important in sperm metabolism and changes in membrane properties must occur for capacitation and the acrosome reaction to occur normally.[8,21] HOST not only assesses the morphologic integrity of the plasmalemma but it also assesses its function and biochemical activity.[8,10] In humans and bulls, there is strong correlation between the HOST, the sperm penetration assay, and there is a good interrelationship between HOST and motility and morphology.[8, 10, 22] In bulls and humans, HOST was a good predictor of success with IVF.[10]

The HOST is simple, fast and inexpensive.[8, 21] One tenth of a milliliter of spermatozoa is incubated in one milliliter of 60 mOsmol fructose solution at 37 °C for 45 minutes. Then 1– 2 drops of this mixture is examined using phase contrast microscopy at 200 x and 400 x. Two hundred sperm are counted and the percentage of sperm with curled or swollen tails is determined. HOST is positively correlated to motility (r = 0.94). The premise being motile sperm have normal membranes and will coil or swell when incubated in a hypo-osmotic solution.

As sperm are cooled for increasing amounts of time, decreasing numbers of sperm will be HOS+ indicating damage to the sperm membrane with prolonged cooling.[21] A similar phenomenon is noted after cryopreservation due to sperm membrane damage. Prolonged heating also damages the sperm membrane resulting in few HOS+ cells. Use of HOST on rewarmed chilled or post-thaw frozen semen may be predictive of the highest quality samples to be used for insemination by selecting for samples with the highest number of HOS+ cells. The HOST may be a beneficial addition to the semen evaluation in dogs with poor fertility but a normal spermiogram.

Acrosome assays, acrosome reaction testing and capacitation testing

To evaluate acrosome status, sperm must be removed from seminal plasma via centrifugation and then are resuspended in capacitation mediim.[23] Hyperactivation can be determined through the use of CASA. The clinical relevance of hyperactivation has yet to be

determined. The acrosome reaction can be evaluated through the use of dyes, fluorescent antibodies or lectins. Induction of the acrosome reaction is most readily induced following incubation of the sperm first in capacitation media and then calcium ionophore (A23187) is added.[8,23] It can be added in high concentration and a short incubation period used (30 – 60 minutes) or at low concentration and a long incubation period used (3 hours). The samples are then washed and re-suspended in protein free media and the cells are smeared on slides that are air dried. The slides are then fixed in alcohol and are stained with peanut agglutinin (PNA), Pisum sativum agglutinin (PSA) or fluorescent-labelled lectins and then evaluated with fluorescent microscopy. The number of acrosome reacted cells are then counted and a percentage of all cells is determined.[23,24] Samples that have high numbers of prematurely reacted cells or which do not respond to incubation with calcium ionophore not likely to be able to complete fertilization.[6,7,23,25,26] In humans, there is a high predictive power of induced acrosome reaction and successful IVF outcome.[26]

Acrosome reaction can also be detected using staining with fluorescein-conjugated lectins, like PSA or PNA, plus fluoresceinisothiocyanate (FITC).[8,25] This combination of stains evaluates damage to the acrosome while at the same time differentiating acrosome reacted from acrosome intact sperm. PSA binds to the acrosomal contents while PNA binds to the outer acrosomal membrane.[27] Acrosomal integrity of canine sperm has been successfully assessed using flow cytometry and staining with FITC conjugated PSA and PI.[28] Capacitation status of chilled and frozen thawed canine sperm has been assessed with a chlortetracycline (CTC) assay and CASA for evidence of hyperactivation.[29] Dog semen has been evaluated for its cryopreservability by first inducing the acrosome reaction with calcium ionophore and then staining the sperm with FITC-PNA along with the membrane impermeable DNA supravital stain ethidium homodimer1 (EthD-1).[30] Samples were evaluated with fluorescence microscopy and flow cytometry. The number of cells that underwent the acrosome reaction via ionophore was well correlated with a similar percentage of cells that had acrosome damage post

cryopreservation. Furthermore, the amount of damage to cells caused by acrosome reaction from calcium ionophore was strongly negatively correlated with the number of motile sperm present after freezing.

Bovine sperm have been incubated with calcium ionophore to stimulate the acrosome reaction and then fixed in formaldehyde.[31] Afterwards they are stained with naphthol yellow S plus erythrosin B or with naphthol yellow S plus aniline blue. This is a permanent fixative and the use of DIC microscopy is required to evaluate acrosomal status. Alternatively, bovine sperm may be treated with fluoresceinated PSA to assess the acrosome reaction similar to that described previously.[27]

Triple staining techniques for acrosomal evaluation have also been described but are more time consuming than the above mentioned techniques and so are not routinely used in the clinical or research setting.[8]

Sperm penetration assays

These assays asses the ability of the sperm to undergo capacitation, the acrosome reaction, membrane fusion and chromatin decondensation in the presence of an oocyte.[4,6-8,23,32] Sperm must be prepared for the assay by incubating overnight in a capacitation medium or storing in a TES-tris buffer with egg yolk for 24 – 48 hours and then applying thermal shock. After this processing step, the sperm are divided into microdrops and zona-free hamster eggs are added. They incubate for 3 hours and the number of eggs penetrated and the number of sperm/egg are counted. The count is performed by looking for swollen heads within the unstained eggs using phase-contrast or phase-interference microscopy or after staining with acridine orange (AO) and using fluorescence microscopy. Use of the TES-tris buffer procedure was more highly correlated with fertility and successful outcomes with IVF in humans in some studies[4,6,7,23] while in others it's predictive power was questionable.[26]

Hemizona assays

This test assesses the availability of the proper molecules on the sperm's head for it to bind to the zona pellucida and initiate interaction with the oocyte.[8,23] A bisected zona pellucida from a normal oocyte is used. Each half of the zona is incubated with sperm for 4 hours and then the number of bound cells is counted. Zona pellucida binding assays have been used to evaluate the fertilizing capacity of chilled and frozen-thawed canine spermatozoa.[33] The test allows an estimation of the damage caused by manipulation of semen on the fertilizing ability of sperm. This test also demonstrates the critical interaction between the zona pellucida and the sperm cell during fertilization and tests multiple sperm functions, including capacitation and ligand-induced acrosome reaction.[4,6-8,34,35] Of the classic sperm parameters, morphology was the best predictor of the ability of sperm to bind to the zona pellucida.[3,4,6,32,34] In conventional IVF studies in humans, defective sperm-zona binding and zona penetration are common causes of failure of fertilization.[7,35] There is a high predictive power of sperm-zona pellucida binding and successful outcome with IVF in humans.[26,35]

Electron microscopy – transmission (TEM)

The sperm rich fraction is mixed 1:2 with cacodylate-buffered 6% glutaraldehyde.[8,36] This mixture is centrifuged, the supernatant removed and the pellet resuspended in 0.1 M sodium cacodylate buffer. This sample is washed a second time and the supernatant removed. The pellet is fixed in a solution of 1% osmium tetroxide in 0.1 M cacodylate buffer and is centrifuged. The osmicated pellet is dehydrated through a graded series of ethanol, is then rinsed in propylene oxide and is then embedded in Poly/Bed 812 or araldite. Sections are cut at 80 nm thickness and are then mounted on 300-mesh nickel grids and stained with uranyl acetate and lead citrate for TEM.

TEM may identify lesions of the plasma membrane, acrosome, mitochondria, and nuclear chromatin.[8,36] Quantification of morphologic defects is not possible but a detailed description of the defects is provided. TEM may also help identify and characterize other cells in

the ejaculate including germ cells, WBC and infectious organisms. DNA fragmentation caused by oxidative stress or exposure to toxins may be identified.

TEM may identify defects of the tail in patients with motility issues that are not apparent with light microscopy.[8,37] If a single defect is present in at least 20 – 30 sections it is considered a ciliary dyskinetic condition. Total or partial dyein arm deficiency occurs in 3% of human patients with abnormal motility. Fragmentation of the plasma membrane and necrosis of the microtubules is typical of necrospermia and is found in 23% of human patients with asthenozoospermia.[38] Multiple fine ultrastructural defects are noted in another 23% of human patients with this condition. Missing outer microtubules, disorganized axonemes, missing central microtubules, additional microtubules above and beyond 9 + 2, thickened and/or disorganized fibrous sheaths, absent radial spokes and translocated tubules are other common defects noted in this group. Some of these defects are noted alone and others in combination. In patients whose total motility is > 30% and at least some normal tails were evident, pregnancies using assisted reproductive techniques (ART) may be successfully obtained.[37]

A microtubular mass defect was noted on examination of spermatozoa from seven stallions with three of these stallions descending from a single sire.[38] There was subfertility in four of these stallions, although it appeared to be at least partially compensable. Detailed description of the ultrastructure of the bovine sperm head and midpiece are available.[39,40]

Electron microscopy is currently available for clinical cases at the veterinary colleges of Auburn University, Texas A & M University, and University of Saskatchewan.

Antisperm antibody assay

In humans, antisperm antibody production is a common cause of male factor infertility.[8,23] There are two commercial assays (SpermMar®, Conception Technologies, and Immunobead Test®, Irvine Scientific, Santa Ana, CA, USA) available for human antisperm antibody assay. These tests provide semiquantitative results regarding the degree of antibody binding present and detect the presence of IgA and IgG antisperm antibodies. If ≥ 20% of the

sperm bind to the beads, a sample is considered positive for the presence of antisperm antibodies.[23] Serum may be assayed for antibodies using a tray agglutination test. At this time, the importance of antisperm antibodies in domestic animals is unclear but may be a useful test for dogs with autoimmune orchitis/epididymitis.

Flow cytometry for DNA and morphology measurement

Binding of fluorescent dyes to sperm chromatin permits the identification of sperm DNA abnormalities and can be measured using a flow cytometer.[8,41] Sperm are stained using fluorescent assay and then run through the flow cytometer to differentiate cells with normal DNA integrity from abnormal. Sperm are typically oriented to be in the same plane before they are excited by a laser beam to induce fluorescence and then flow past a fluorescence detector which monitors exactly how much fluorescence each cell has. Sperm that have uniform head size and shape display a uniform degree of fluorescence while cells with abnormal size and shape have amounts of fluorescence outside the normal ranges.[41] In addition to being able to differentiate morphologically normal from abnormal sperm, flow cytometry can also differentiate sperm with normal motility from those with decreased motility.[42] One cause of decreased motility in humans is a result of a break in the DNA strands of the sperm nucleus and their mitochondria. Flow cytometry and TUNEL (terminal deoxy-nucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labeling) testing both confirm the presence of these strand breaks in this population of asthenozoospermic men.

One of the greatest assets of using flow cytometry for sperm evaluation is the sheer number of sperm that can be evaluated in a short period of time. Routine microscopic assessment of sperm involves counting either 100 or preferably 200 cells. But with the use of flow cytometry, thousands of cells can be assessed in a matter of minutes.[41] Another assessment that can be performed using flow cytometry is that of sperm viability. A dual staining technique using carboxifluorescein diacetate (CFDA) and propidium iodide (PI) was used to validate the ability to differentiate live canine sperm from dead sperm.[43] Plasma

membrane integrity was also validated in stallion sperm using either CFDA and PI or SYBR-14 stain and PI.[25] In these tests, live sperm fluoresce green (from CFDA or SYBR-14), dead sperm fluoresce red (PI), while dying sperm fluoresce both colors.[25,32]

Tests of mitochondrial activity

Rhodamine 123 (Rh123) is a mitochondrial probe which is combined with the viability stains of PI and carboxydimethylfluorescein diacetate (CDMFDA) to allow for the determination of sperm with intact membranes (CDMFDA+) and functional mitochondria (Rh123+) from dead sperm (PI+).[25] Stallion sperm with functional membranes and mitochondria correlate well with sperm viability and motility.[25]

Cytochemical tests for sperm chromatin integrity

The integrity of nuclear chromatin results from a combination of factors including sperm maturation processes, damage due to oxidative stress and other endogenous factors.[8,44] Chromatin maturation depends on proper replacement of histone with transition proteins and finally basic protamines. This transition results in compact packaging of the DNA and is enforced by cross-linking with protamine-disulfide bonds. In some abnormal sperm, histones may be partially or completely replaced by protamines resulting in loosely packed chromatin. Detection of this loose packing may be found with the aniline blue (AB) test. DNA is more predisposed to denaturation by heat and low pH when chromatin is packed loosely than when it is tightly packed.[44] Chromatin proteins in sperm with abnormal DNA are more susceptible to staining with acidic dyes like AB, acridine orange and toluidine blue.[44] All three of these staining protocols provide a good estimation of the number of sperm with normal vs loosely packed chromatin structure. If more than 30% of sperm have loosely packed chromatin structure an association with increased infertility is noted which correlates with results of SCSA (see below).

Sperm chromatin analysis

Sperm morphology is well correlated with semen quality.[4,7,8,45,46] The head of the sperm consists primarily of nuclear chromatin, so subtle changes in sperm head morphology may be

related to abnormalities of DNA content. Measurement of a set of parameters regarding the sperm head (size and shape) and midpiece can align sperm into certain populations of sperm, such that the chromatin content in each sperm head can be determined to be normal or abnormal using automated sperm morphometric analysis (ASMA). Non-compensable defects (those that cannot be overcome by increasing the number of sperm in a breeding dose) are typically related to sperm with abnormal head morphology.[4,7,45,46] Sperm head shape has been correlated with fertility and resistance to cryopreservation. Sperm nuclear DNA fragmentation is positively correlated with lower IVF fertilization rates, impaired implantation, increased risk of abortion, and increased risk of disease in offspring, including pediatric cancer.[7,8,31,47-49,51] Fertile sperm have stable DNA which is able to decondense at the appropriate time during the fertilization process such that the oocyte has access to this DNA for combination with its own DNA complement.[48,49,52]

Sperm DNA damage may occur on several different levels.[4,7,8,31,48- 50,52] Mitochondrial DNA damage can occur and be manifest as deletions, point mutations and polymorphism and is associated with decreased semen quality, asthenozoospermia and male infertility. Nuclear DNA damage may occur as a result of oxidative stress, sperm chromatin packaging and apoptosis. DNA damage may occur as a result of environmental factors, pollutants, infection, inflammation, or the presence of ROS.[48,49]

For SCSA, sperm are treated with an acidic solution (pH1.2) in order to denature their DNA in situ.[8,46,48] Sperm with normal chromatin will not denature under these conditions while abnormal DNA will denature. The sperm are then stained with acridine orange. This is a metachromatic DNA stain. Chromatin which has been denatured into single stranded DNA will fluoresce red, while DNA which does not denature (remains double stranded) will fluoresce green. The percentage of cells with denatured DNA is determined and is called %COMP. The percentage of sperm with non-detectable vs detectable DNA fragmentation is called the DNA fragmentation index (DFI). The percentage of sperm with immature chromatin is called high

DNA stainability (HDS). Combining the use of SCSA and ASMA may assist in evaluating dogs with normal spermiograms and poor fertility.[46,48]

DNA fragmentation may also be evaluated using alkaline single-cell gel electrophoresis testing, TUNEL assay; Comet assay, in situ nick translation, and DNA breakage detection-fluorescent in situ hybridization assay (DBD-FISH). These assays use fluorescence microscopy.[7,48-50,52] Staining techniques using aniline blue, toluidine blue and chromomycin A3 also may be used to identify chromatin packaging defects.[52] At this time, few of these assays beyond SCSA are used in clinical practice as it remains to be determined what the clinical relevance of negative outcomes means to fertility. There also still remains significant variability in techniques between labs resulting in disparate results. Certainly in veterinary medicine, data regarding most of these tests in clinical practice is lacking, although they are slowly being introduced.[32]

On the other hand, SCSA, has been accepted as an important tool in the diagnosis of infertility and in prognosticating human couple's success rates with ART.[48,49] In humans, DFI is a strong indicator of successful pregnancy outcome.[4,7,48-50] Prediction of successful outcome with intrauterine insemination is well correlated with the degree of DNA fragmentation.[48,49] The number of sperm that have DNA strand breaks is negatively correlated with their ability to fertilize an oocyte during IVF.[48-50] Sperm DFI was shown to be negatively correlated with sperm concentration, motility and normal morphology.[50] Fertilization failure of sperm with DNA fragmentation may be able to be overcome by application of ART procedures, such as intracytoplasmic sperm injection (ICSI), however embryo development may be affected with resultant early embryonic death (EED) or abortion.[4,7,32,48-52] In humans, a DNA fragmentation rates of > 30% seem to impede fertility, and in couples with high DFI, the use of ICSI will improve pregnancy rates over the use of IVF.[48,49,52]

In stallions, SCSA has been evaluated and shown to be an indicator of some forms of subfertility or infertility.[25,53] Subfertile stallions had higher %COMP levels than did stallions with

normal fertility and there was a negative correlation between seasonal pregnancy rate and %COMP, % morphologically normal sperm, and % motile sperm. In boars, there is an inverse relationship between farrowing rate and numbers of pigs/litter compared to %DFI.[48] Pregnancy rates in bulls with high %DFI were lower than for bulls with low %DFI.[48] It appears that the threshold for fertility in bulls (10 – 20%) and boars (8%) is much lower than for humans).[48] A threshold level is not yet available for dogs.

SCSA is currently offered for clinical cases at SCSA Inc (Brookings, SD; www.scsa.com) and at Texas A & M University College of Veterinary Medicine.

Assays for reactive oxygen species

ROS are very important during the sperm capacitation process in order for tyrosine phosphorylation events to occur normally.[7,8] They are normally produced at low levels, however, in some cases of infertility they are produced in much higher amounts. ROS interrupt sperm function by causing peroxidative damage to the plasma membrane and thereby impairing motility, the acrosome reaction (exocytosis), and disrupting sperm-oocyte fusion. Oxidative stress may also cause mitochondrial DNA and nuclear genome damage.[7] There are chemluminescent assays using redox-sensitive probes (lucigenin and luminol) for human spermatozoa.[7,8] High levels of chemluminesence affect the fertilizing capacity of sperm both in vivo and vitro. The presence of WBCs in the ejaculate will greatly increase the amount of ROS present, therefore WBCs must be removed from the samples prior to testing.[7] In the presence of seminal plasma, protection from ROS produced by WBC is afforded, while for ART technologies, these ROS will likely have a much more significant role.

Fluorescence in situ hybridization (FISH)

This procedure allows for the analysis of chromosome numbers in individual sperm. Individuals with oligoasthenoteratozoospermia are at increased risk of having chromosomal abnormalities such as aneuploidy, double aneuploidy and diploidy.[1,54] Sperm are fixed in a methanol:acetic acid solution and then the DNA is decondensed in an acidic salt solution.

Sperm probes for specific chromosomes each fluoresce in specific color ranges are applied and in situ hybridization is performed. In humans, teratozoospermia in the form of macrocephalic, multi-tailed sperm have an increased incidence of aneuploidy.[54] Other morphologic abnormalities may also be associated with specific chromosomal abnormalities and this area bears the need for further investigation.[54]

Computer assisted sperm analysis and automated sperm morphometric analysis

CASA is a technique employing a computerized system that tracks mean percentage of motile sperm, mean percentage of progressively motile sperm, mean curvilinear velocity and mean straight line velocity per microscopic field.[8] The ejaculate is diluted to a specified concentration and the system uses a special gridded microscope slide that accepts a constant volume of semen thus providing a consistent number of sperm to be evaluated each time. The pre-warmed and loaded slide is placed into a thermostatically controlled chamber for analysis. The computer takes video images of the sperm and stores then for analysis. The system recognizes motile from non-motile sperm and other organic debris by comparing luminosity (gray-scale intensity) and size of the object. There are also preset user-defined thresholds for size and luminosity that help prevent mistaking other cells and debris for non-motile sperm. Computerized systems have been shown to be more accurate than subjective assessment of sperm motility in human, equine, bovine and canine studies.[6,8,32,55-57] CASA provides a more discriminating estimation of motility than subjective evaluation with greater repeatability.

ASMA is now also available and provides a more accurate and repeatable evaluation of general sperm morphology.[4,6,25,32,46,58,59] With this automated process, as with CASA, sperm are diluted in physiologic media to a specified concentration and then a fixed drop placed on a slide and air dried. The slides are then stained and coverslips permanently affixed to the slide before processing in the analyzer. In this way a consistent number of sperm/field may be analyzed. Staining method is also important. Papanicolaou stain and Giemsa stain have been used successfully for morphometric analysis in humans, stallions and dogs. The machine obtains a

variety of head measurements including length, width, area, perimeter and width/length. A specified gray scale is required and allows differentiation of sperm heads from other cells and debris on the slide. Midpiece width and area, distance between the major axes of the head and midpiece, angle of divergence of the midpiece from the head axis can also be assessed. Abnormal sperm head, midpiece and tail morphology can be detected 95% of the time with these measurements.[45,46,58]

In the normal dog, a significant variation in head area, length, width and roundness exists, but the analyzer still provides accurate differentiation of teratozoospermic samples.[45,46,59] Ovalness was the least variable factor obtained while length and width had more variation between dogs. Within dogs, there was less variation of any measurement. Similar variability in sperm head shape and size is noted with SCSA analysis of the same dogs, indicating that ASMA may be a valuable tool when assessing sperm for teratozoospermia.[46]

In vitro fertilization

This is the ultimate test of sperm function.[8,23] In human medicine, the end point of IVF is what percent of MII oocytes are fertilized and develop to the 8 cell stage by day 3 post insemination.[4,32]

Sperm function tests

Table 1. Sperm functions and the sperm function tests that assess them[23]

Motility	CASA	Light microscopy		
Morphology	ASMA	Light microscopy	EM	Flow cytometry
Capacitation	IVF	SPA		
Acrosome Reaction	IVF	SPA	Acrosome reaction tests	Acrosin assays

Zona pellucida binding	IVF	HZA		
Zona pellucida penetration	IVF			
Oocyte-sperm fusion	IVF	SPA		

CASA – computer assisted sperm analysis; ASMA – automated sperm morphometric analysis; EM – Electron microscopy; SPA – sperm penetration assay; IVF – in vitro fertilization; HZA – hemi-zona assay

Summary

It is clear that there is much more to be evaluated regarding male fertility than the basic semen evaluation. The concept that simply providing a specified number of normal appearing, motile sperm at the proper time in relation to breeding will result in acceptable pregnancy rates is clearly a misconception. There are many aspects of sperm function that may affect the functional competence of the sperm cell, beyond its basic size, shape and motility.[7] When faced with a dog that has subfertility, the clinician must first rule out the most obvious causes for the problem and them move on to more advanced semen diagnostic testing to exhaust all possible diagnoses. In the process of making the diagnosis, the clinician may discover a method of treating or correcting for the problem. Unfortunately, in some cases, even with exhaustive testing, a diagnosis may remain elusive. Research in all areas discussed in this paper is needed for all domestic animals including the canine.

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