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Review Article

COLLECTION AND EVALUATION OF CANINE SEMEN-A REVIEW

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Abstract: Compared to human medicine, little is known in canine medicine regarding specific findings on semen evaluation and theircorrelation with fertility. Semen collection and evaluation are essential skills for small animal practitioners. Several tests including motility in bright field and phase contrast microscopy, sperm morphology, sperm membrane integrity, capacitation and sperm function tests have been investigated to predict fertility. The fertility of the female also plays a crucial role in estimating the fertility of the male. Poor female fertility can make a fertile male appear less fertile. Therefore, the aim of the present review is to discuss several recent techniques for the assessment of canine semen quality.

Keywords: Dog; Spermatozoa; Evaluation; Viability; Fertility.

INTRODUCTION

The ultimate goal of semen evaluation is to predict the fertilizing capacity of a semen sample. Generally, males with optimal fertility produce semen with high number of progressively motile, viable and morphologically normal spermatozoa. Whereas infertile or subfertile individuals contain low percentage of normal viable sperm. Till date, light microscopy is routinely used to evaluate the principal parameters of semen,viz. concentration, motility and morphology. Concentration is usually determined using a Neubauercounting chamber. Motility is assessed subjectively on a pre-warmed glass slide (Johnston, 1992; Iguer-ouada and Verstegen, 2001), whereas morphology defects are assessed with various staining techniques (Oettle, 1993; Johnston, 1992). Recently, several techniques have been described which may enable more accurate prediction of the fertilizing capacity of canine spermatozoa. This allows assessment of many functional characteristics of spermatozoa which are related to the binding, penetrating and fertilizingcapacity of an oocyte (Van Soom*et al*., 2001; Hewitt and England 2001).

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SEMEN COLLECTION

The most commonly used method of semen collection in dogs is manual. Sperm can also be collected from the epididymis after surgical sterilization, post-mortem or vaginal lavage after natural mating (Axnér and Linde-Forsberg, 2002).

A teaser bitch is used for semen collection. Preferably bitch should be in proestrus or oestrus and weight should be equal to that of male dog. Vaginal secretion swab of proestrus/oestrus bitch, which is applied on the vulvar area and tail head of an anoestrus bitch (Seager, 1986). Sexual rest for 4 to 5 days is ideal as compared to sexual rest of more than 10 days, that can cause increased morphological abnormalities, decreased motility due to spermatozoal aging and increased debris (Purswell*et al*., 1992; Johnston *et al*., 2001).

An assistant should restrain the bitch at the end of the rubber-backed mat, with her tail toward the mat. If bitch is furious always muzzle should be used. An inexperienced or nervous dog can be allowed to sniff and play with the bitch before collection (Schubert and Seager, 1991). Lubricating agent is not recommended during semen collection (England and Allen, 1992; Freshman, 2001). While the dog is sniffing the bitch's perineal area, the semen collector kneels or squats on the left side of the dog (reverse for left-handed collector). The collector's right hand efficiently massages the prepuce over the penis. The collector's left hand holds the collection cone with attached collection tube at the tip of the prepuce. As the dog's penis reaches 40 to 50% erection, the prepuce is pushed behind the bulbusglandis with the collection cone. A circular, firm pressure should be maintained with the left hand to simulate the copulatory lock or tie. (Freshman, 2001; Johnston *et al*., 2001; Seager, 1986). Continue to apply pressure until crystal clear fluid (prostatic fluid) begin to flow into the collection tube.

If the bulbusglandis has enlarged so that the prepuce cannot be moved behind it, take the dog away from the bitch and allow his erection to subside before trying again. Complete erection and ejaculation while the bulbus is within the prepuce can be painful and results in an incomplete collection (Freshman, 2001).

After collection, the bitch is removed from the room. The bulbusglandis should be lubricated, which will help the penis return to its normal position. Lubrication applied at the junction of the prepuce and penis, which minimizethe entrapment of the tip of the penis by the distal preputial skin rolling inward. The dog should not be placed with other dogs until his penis is back within his prepuce.

Fractions of Semen Ejaculation

Dogs ejaculate in three fractions. The first fraction is termed as presperm fractionwhich originates from the prostate gland. Normally it is clear or slightly cloudy and volume ranging from 0.5 to 20 ml or more (Feldman and Nelson, 1996; Freshman, 2001). The second fraction is called as sperm-rich fraction which is normally opaque, milky-white in color and ranging from 0.5 to 2.0 ml. This fraction comes from storage in the tail of the epididymis, as well as from daily sperm output (Johnston, 1989). The first and second fractions are often collected together and are ejaculated during and immediately after the dog's vigorous thrusting movements (Feldman and Nelson, 1996). For chilled or frozen semen collection and some diagnostic test these two fractions should be separated. Contact of spermatozoa with first or third fraction fluid may decrease motility after two hours of collection (England and Allen, 1992). The third or prostatic fraction is normally clear and may consist of more volume, depending on how long pressure is maintained proximal to the bulbusglandis (Johnston, 1989). This prostatic fraction is also useful for evaluating the prostate diseases.

EVALUATION OF SEMEN

The spermatozoa, is a complex and very specialized cell adapted to transport the male genome to the female genital tract to fertilize the oocyte. Because of its complexity, a simple test cannot give complete information of the fertilizing potential of a sample and different tests are necessary (Ioana*et al.*, 2012).

Colour:

A clear semen sample contains no spermatozoa. Cloudy or milky samples probably contain spermatozoa but always should be checked microscopically for confirmation. Occasionally, a dog with azoospermia will shed excessive numbers of fat droplets into the samplewhich giving the appearance of normal semen.Yellow colour can indicates the urine contamination. Green colour denotes presence of pus. Red or brown colour indicates fresh or haemolysed blood in semen (Johnston *et al*., 2001). The most common causes for blood in the semen include prostatic disease or damage to blood vessels on the penis. Presence of blood in the semen has no effect on motility of spermatozoa until six hours of contact (England and Allen, 1992). The hemospermia indicates prostatic disease and penile trauma in dogs (Root Kustritz, 2007).

Volume:

The volume of semen is not an indicator of semen quality in dogs. However, the volume should be measuredas the part of the calculation of total number of spermatozoa in the

sample. The volumeof the first and third fraction especially the latter are variable and the volume is controlled by the person collecting the sample.

pH:

Dog seminal plasma has a normal pH ranging from 6.3 to 7.0. Threlfall (2003) recommended that evaluation of pH be performed immediately after collection using accurate equipment (presumably a pH meter) and strongly discouraged use of a ‘‘dipstick’’ method. Prostatic fluid has a normal range of 6.0 to 7.4 with an average of 6.5 or 6.8. (Feldman and Nelson, 1996; Johnston, 1989). The pH is being helpful for antibiotic selection in case of infection.

Motility:

Motility is a manifestation of structural and functional competence of spermatozoa; thus, the percentage of progressively motile spermatozoa is usually positively correlated with that of plasma membrane integrity (Kumi-Diaka, 1993; Rodriguez-Gil *et al*., 1994) and of normal morphology (Ellington *et al*., 1993). Spermatozoal motility should be evaluated immediately after collection. If the sample is too concentrated to evaluate motility a drop of semen can be diluted with a drop of buffered saline solution at the appropriate pH (Johnston, 1989). Recent information indicates a warmed slide may not be required because canine semen is resistant to cold shock, at least above 70 ° F (Johnston *et al*., 2001). Normal motility is described as rapid, progressive, forward motion. (Feldman and Nelson, 1996). The percentage of total motile spermatozoa in normal canine ejaculates is between 70 to 90% (Johnston *et al*., 2001; Iguer-Ouada and Verstegen, 2001). Although, it may be lower after prolonged periods of sexual rest. It has been proposed that fertile dogs should have at least 70% of total sperm motility (Larsen, 1980). Speed or quality of motility also may be assessed; a canine spermatozoon with normal motility should traverse the microscopic field of view in 2–3 sec (Threlfall, 2003).

Morphology

The wet mounts may be useful to detect sperm defects of mid piece and tail. However, head defects, tail defects and acrosomal defects may be better evaluated with stained smears. Abnormality is evaluated by counting about 100 to 200 spermatozoa in a stained semen slide under 100× (oil immersion). Count only free heads and not free tails (Feldman and Nelson, 1996). Abnormalities can be classified as primary (occurring during spermatogenesis) or secondary (occurring during maturation or sample preparation) or as major or minor abnormalities (Oettle and Soley, 1988). Normal semen samples should have <10% primary

abnormalities and <20% secondary abnormalities. Total abnormalities should be <10-20% (Feldman and Nelson, 1996; Freshman, 2001; Purswell*et al*., 1992).

Concentration

Concentration of sperm has little value as an indicator of semen quality. The normal total number of spermatozoa in dog semen is greater than 300 - 2000 million (Johnston, 1992). Concentration is inversely related to volume of semen collected. Total number of spermatozoa is dependent on testicular size (Olar*et al*., 1983) and it may decrease with frequent semen collection, presumably as epididymal reserves are depleted (England, 1999). Normal dogs may ejaculate oligozoospermic or azoospermic samples due to anxiety or pain (Martinez, 2004).The traditional technique for assessing spermatozoal concentration was done with the use of haemocytometer. The hemacytometer technique has been reported to be equally accurate or more accurate than CASA systems and its considered the gold standard. Spermatocrit is determination of concentration by evaluation of the percentage of solids when semen is centrifuged in a haematocrit tube; this technique is not accurate in dogs (Root Kustritz*et al*., 2006).

Live-dead staining

Eosin–nigrosin is one of the best method to determine the percentage of live-dead sperm in given samplewhich is most widely used in all domestic species. It is simple, rapid and does not require cell manipulation in order to determine the proportions of normal and abnormal, live and dead spermatozoa. Live spermatozoa with intact acrosomes appears to be white against the dark background of nigrosin exhibiting a regular and well defined apical ridge. Good canine semen sample should contain at least 80% morphologically normal and viable spermatozoa (Johnston *et al*., 2001). When the proportion of normal spermatozoa was below 60 percent, fertility was found to be adversely affected (Oettlé, 1993). Problems with this test include inability to classify spermatozoa with partial staining and interference with staining if glycerol or fat globules are present in the seminal fluid (Rijsselaere et al., 2005).

Fluorescent staining

Fluorescent staining is used to evaluate integrity of the plasma membrane and capacitation status in canine spermatozoa (Rijsselaere *et al*., 2005). Fluorescent stained sperm can be assessed for functional and morphological aspects without interference of the extracellular media. Many different fluorescent staining and combinations of fluorophores have been developed to evaluate different sperm characteristics in several mammalian species, which may be analysed by fluorescence microscopy or by flow cytometry: proportions of live and

dead cells, mitochondrial function, acrosomal integrity, capacitation status, intracellular calcium concentration of spermatozoa, sperm chromatin structure and DNA content (Martinez, 2004).

Hypo-osmotic sperm swelling (HOS) test

The HOS test involves submersion of spermatozoa into a hypo-osmotic medium. Those spermatozoa that have intact plasma membranes will swell as fluid moves into the sperm cell; this will cause swelling and coiling of the tail (Martinez, 2004). Hypo-osmolar solutions described include sodium citrate (7.35 g) and fructose (13.51 g) in 1000 mL of distilled water, and 100 mM sucrose solution (Inamassu *et al*., 1999; Pinto *et al*., 2005). In one study, maximum percentage of swollen spermatozoa was observed after 45 - 60 min of incubation at 37.8°C in the hypo-osmotic solution. In another study, there was no different in percentage of swollen spermatozoa between those samples incubated for <1 min and those incubated for 60 min (Rodriguez-Gil *et al*., 1994; Pinto *et al*., 2005). Remember to evaluate the percentage of spermatozoa with coiled tails before performing the HOS test; the initial value must be subtracted from the percentage of spermatozoa with coiled tails after incubation to get the true percentage of spermatozoa with presumed intact plasma membranes as determined by this test (England and Plummer, 1993; Kurni-Diaka and Badtram, 1994; Inamassu *et al*., 1999).

Cytology

Cytology of the sperm-rich and prostatic fractions should be evaluated separately. One way is to centrifuge 0.3 to 0.5 mL of sample at 120g for 7 min. The slide is preparedwith the pellet and stained with Diffquick stain. Normal cytology of the sperm-rich fraction contains spermatozoa, white blood cells (WBC), red blood cells (RBC), epithelial cells and bacteria. Prostatic fluid also contains epithelial cells, WBC and bacteria (Johnston*et al*., 2001). Increased or degenerate WBC or intracellular bacteria indicate infection. IncreasedRBCs may indicate prostatic disease or bleeding from penis or prepuce. (Seager, 1986). Presence of inflammatory cells is not well correlated with lack of significant bacterial growth from semen samples in dogs; 44% of dogs with no inflammatory cells in their semen had significant aerobic bacterial growth from that semen in one study (Root Kustritz *et al*, 2005).

Semen Culture

Semen is not sterile. A wide variety of normal flora is present in semen. Before collection, clean the prepuce and tip of penis with sterile saline moistened gauze (Feldman and Nelson, 1996). Culture of the distal urethra to compare flora may also be useful. Greater than 10,000

CFU of aerobic bacteria per mL of semen indicates infection. Anaerobes are not common in canine semen. Mycoplasma is a normal finding in the distal canine reproductive tract. However, it can cause the problems if present in excess amounts.

Filtration

Filters may be used to assess for normal function of spermatozoa by evaluating motility via determination of extent to which spermatozoa can penetrate the filter or by binding abnormal spermatozoa. While percentage of morphologically normal spermatozoa (MNS) may be much better in semen after filtration. Total number of spermatozoa present in the filtrate may be much lower than that in the original sample. In a study, evaluation of semen was done in four dogs before and after passage through a glass-wool filter. The average concentration of spermatozoa in the unfiltered samples was 445.7 million/mL and after filtration it was 53.3 million/mL (Mogas *et al*., 1998).

Alkaline Phosphatase (ALP)

ALP is produced from the epididymis. This makes it an excellent marker for patency of the ductal system.In normal semen samples, the ALP has a range of 5,000 to 40,000 U/L (Johnston, 1989).

Centrifugation

In this method, the abnormal spermatozoa and other cells are bound which accumulate as a pellet in the bottom of the tube and normal spermatozoa can be moved through it. Canine spermatozoa have been demonstrated to be not damaged by centrifugation. This technique greatly improves percentage of MNS in the centrifuged samples. Digested and fresh blood also may be removed by using this method (Root Kustritz, 2007).

Evaluation of sperm function using in vitro gamete interaction tests

Hemi-zona, zona pellucida binding and oocyte penetration assays have been developed to investigate the fertilization process in dogs (Hewitt and England, 2001) to discriminate between fertile and subfertile dogs (Mayenco-Aguirre and Pérez-Cortés, 1998) or to evaluate the effect of different preservation methods on the fertilizing capability of dog spermatozoa (Hay *et al*., 1997). These systems can provide valuable information on the fertility potential of a male and the efficacy of a semen preservation method being tested.

CONCLUSIONS

Semen collection and evaluation is a valuable service that can readily be performed. Attention to the dog's comfort and the presence of a teaser bitch maximize the semen collection quality. Multiple collections may be needed to truly evaluate the dog. Several new

techniques have been introduced which allow for a very detailed and rapid evaluation of various specific functions of sperm. A combination of several sperm quality measurements probably has a better correlation with fertility than any single measurement. The high incidence of conception failure is due to poor breeding management and mistimed breeding.

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