Canine artificial insemination with fresh, refrigerated and frozen semen  
[Inseminação artificial na cadela com sémen fresco, refrigerado e congelado]

Stefano Romagnoli

Introduction

Artificial insemination (AI) in the dog is commonly practiced when the female does not accept breeding by a specific male, when a male cannot mount due to physical problems (vertebral column disease, hind leg disease, excessive panting in brachicephalic breeds etc.), or when male and female live far apart and cannot travel. Semen collection in the dog is a simple technique which requires very little training and which can provide veterinarians with extremely important clinical information concerning the present and future fertility of their canine patients.

Semen collection

Equipment: latex cone (not indispensable); 10-15 cc sterile tubes; bitch in heat (not indispensable); latex gloves; microscope with a 100x ocular; microscope slides; Pasteur pipette; micropipette or insulin syringe; formaline; haemocitometer; stain (eosine-nigrosine, Diff-Quick etc).

Collection technique: Massage the bulbus glandis until partial erection is achieved, then push back the preputium and insert the artificial vagina (latex cone) or grasp the bulbus glandis with a gloved hand and keep massaging the penis; when the dog lifts his back leg push the penis backward rotating it of 180°. Canine semen is ejaculated in 3 fractions: the first is prostatic fluid (only a few drops which are generally expelled while the dog attempts to mount the female); the second is the spermatic fraction (0.5-3.0 cc depending on testicular volume), the third is prostatic fluid (4-50 cc depending on prostatic volume). Collect the first and second fractions for semen evaluation, collect the first+second and a 1-2 cc of the third fraction to perform an artificial insemination. Prostatic fluid is generally not collected for semen freezing or refrigeration (if in excess it may be discarded by centrifugation), while it can be used for fresh semen artificial insemination provided that it is clear, transparent and shows no signs of pathology.

Semen Evaluation

Sperm motility, concentration and morphology, sediment, presence of white blood cells in the sperm sediment, seminal plasma pH and alkaline phosphatase should be assessed. Sperm concentration can be measured loading a haemocytometer with the right amount of semen and the right dilution rate according to manufacturer’s instruction for the haemocytometer being used. The number of cells in the central square millimetre x 10^6 x semen volume gives the total number of spermatozoa/ejaculate (normal is 400-2000 million sperm/ejaculated). Use formalin to dilute semen when loading the haemocytometer; water can also be used but it will cause high percentage of proximally coiled tails. Sperm morphology (normal is < 20% pathologic spermatozoa) should be evaluated on at least 100 cells (we normally evaluate 200 sperms in infertility cases). Sperm sediment is generally absent, or there may be rare epithelial cells from the reproductive tract; Sperm bacterial culture should yield a count of ≤10,000 colony forming units/ml, negative for Mycoplasma and Brucella canis. A practical way of doing it is to aspirate 4 aliquots of 0.1 cc of semen in 4 syringes, submitting one for aerobic, one for anaerobic, one for mycoplasma and one for B. canis culture. The number of white blood cells (WBC) can be calculated by counting the number of WBC in the 4 large corner squares of the haemocytometer and multiplying by 250 (normal is < 2000/ml). Seminal plasma alkaline phosphatase (ALP) is produced in the epididymides. Low concentration or absence of ALP indicates incomplete ejaculation or bilateral obstruction of the...
epididymides or of vasa deferentia. Seminal plasma ALP can be measured using laboratory equipment routinely employed to measure the enzyme in serum. Laboratory technicians should be advised to centrifuge the semen sample (some sophisticated equipments may be damaged by spermatozoa) and also to dilute the centrifuged sample as seminal plasma ALP concentrations are typically very high (5,000-40,000 IU/L), and the result of the undiluted sample could be so high that might not be readable.

In order for canine artificial insemination to be successful it is important to a) identify the day of ovulation, b) use semen of good quality, c) use a good technique of semen dilution and preservation, d) use a proper technique of insemination.

**Timing ovulation in the bitch** – Canine proestrus and estrus last on average 9 days each with ovulation taking place 3 days after onset of estrus (or day 12 after onset of proestrus). However ovulation can occur as early as 5 days or as late as 27 after onset of proestrus. Therefore, it is very important to check the female’s behavior, perform vaginal smears every 2-3 days starting on the first day of proestrus in order to catch early ovulators, and draw blood samples to measure progesterone once behavior and/or vaginal smear indicate estrus. Estrus is indicated by acceptance of the male or by a degree of vaginal cornification of ≥70. Ovulation occurs 3 days after onset of estrus. Serum progesterone has a concentration of (values are approximate) 2.0-3.0 ng/ml on the day of the peak of luteinizing hormone (LH), 4.0-8.0 ng/ml on the day of ovulation, 10-25 ng/ml during the 2 days following ovulation, which is when oocytes are reaching maturity in the ampullae of the oviducts and fertilizations are taking place. Ovarian structures can be visualized with ultrasound using 5.0 to 7.5 sectorial MHz probes; follicular growth can be followed and ovulation can be estimated based on disappearance of the hypoechochogenic areas representing follicles (which become luteinized) and on appereance of an hypoechochogenic area at the periphery of the ovary representing follicular fluid accumulation within the ovarian bursa.

**Fresh and Refrigerated Semen** – In most countries of the world canine AI is performed using fresh semen. When properly performed, the success of AI with fresh semen is equal to the success of natural breeding, i.e. ≥80%. Although shipment of fresh undiluted semen can be done provided that travel time does not exceed few hours (and provided also that prostatic fluid is normal), it is always better to dilute semen as spermatozoa lose very rapidly their fertilizing ability when maintained in seminal plasma. Semen extenders protect the sperm membrane from temperature variations as well as from mechanical trauma during transport, providing also stable pH and temperature conditions. Antibiotics such as streptomycin and penicillin should be use especially when storage is prolonged for more than a few hours especially when using egg yolk-based extenders where bacterial growth is enhanced. Table n° 1 shows the recipes for 2 canine semen extenders, one rather simple, milk-based extender good for a practice situation which maintains normal semen motility for 24-36 hours and a more complex, Tris-fructose-egg yolk based extender which requires more time to be prepared but allows a semen survival of up to 4-5 days. Properly extended and refrigerated semen placed in a plastic vial can be shipped across countries in a thermos.
Canine semen should be diluted 1:3 or 1:5 depending on its concentration. If the semen sample is too diluted it can be centrifuged at 500 g for 5 minutes to remove the excess prostatic fluid prior to adding the extender. Refrigeration can be performed in a normal refrigerator for 30-60 minutes, after which the semen sample can be shipped in a thermos. Keep in mind that a motile semen sample may already have lost its fertilizing capacity: it is generally believed that motility lasts for about twice as long as fertilizing ability. Prior to insemination let the semen sample reach slowly room temperature.

Frozen Semen. Canine semen freezing techniques are not too complicated but are fairly elaborate and require long hours of labour as well as a great degree of attention to details. The topic has been extensively reviewed in the literature (Concannon and Battista, 1989; England, 1993). Canine semen can be frozen rather simply pouring liquid nitrogen in a styrofoam box, exposing paillettes to liquid nitrogen vapors and then plunging them directly at –196 °C. However, due to the high variability in the response of canine semen to freezing and thawing, such simple “exposing and plunging” technique does not always give satisfactory results. While the process of thawing frozen semen and performing an artificial insemination can be a good investment and a source of professional gratification for private practitioners, freezing semen requires too much time and labour and too big an investment for it to be considered rewarding from a practice point of view.

Freezing extenders generally contain glycerol as a cryoprotectant. Thawing protocols vary and should be consistent with the freezing protocol, therefore it is advisable to check with the laboratory where the semen was frozen. In general, canine semen frozen in 0.5 cc paillettes (paillettes are better than pellets for the dog) should be thawed in a 37 °C water bath (made of water or a thawing solution such as saline or sodium citrate) for 15 seconds. A faster thawing can be performed at 35°C for 30-120 seconds, 75° C for 6.5, 8 or 12 sec.; 75° C for 5 sec. for 0.25 ml (mini) paillettes.

Fertility following use of refrigerated or frozen semen AI is generally lower than fertility of natural breeding because a) ovulation timing is often not properly done (especially considering that frozen-thawed semen lasts only 2-3 days, which means that finding the right time frame for insemination becomes truly critical); b) if refrigeration is not well performed semen may actually be damaged; also, for some dogs the freezing-thawing process is highly detrimental and their semen quality becomes very poor; c) the population of female dogs on which AI is performed is often “skewed” towards infertility, i.e. it is likely that infertile bitches are overly represented in such a group. Data from Sweden indicate that conception rate following use of refrigerated and frozen semen is 54.7% e 39.0%, respectively, while if only bitches in which ovulation well was timed are included conception rates are 62.3% e 51.1%, respectively.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Tris-egg yolk</th>
<th>Skim Milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>3.026 g</td>
<td></td>
</tr>
<tr>
<td>Citric acid</td>
<td>1.7 g</td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>1.25 g</td>
<td></td>
</tr>
<tr>
<td>Skim milk</td>
<td>100 ml</td>
<td>100 ml</td>
</tr>
<tr>
<td>Sodium Penicilline</td>
<td>100.000 UI</td>
<td>100.000 UI</td>
</tr>
<tr>
<td>Streptomicine</td>
<td>100 mg</td>
<td>100 mg</td>
</tr>
<tr>
<td>Bidistilled water</td>
<td>fino a 100 ml</td>
<td></td>
</tr>
<tr>
<td>Egg Yolk</td>
<td>20% v/v</td>
<td>20% v/v</td>
</tr>
</tbody>
</table>

Table n° 1 – Composition of two canine semen extenders: a rather elaborate one which prolongs motility for up to 5 days (tris-egg yolk), and a more simple, practice oriented extender which can only be used for short shipments (skim milk, 24-36 hours).
Insemination Technique. Fresh semen can be deposited in the cranial portion of the vagina through a plastic catheter. Rigid catheters used for large animal uterine flushing work well although they need to be shortened for the use in bitches. Intravaginal insemination is easy and widely practiced and conception rates following use of fresh or refrigerated semen are good. Ideally the bitch should have an empty stomach (not indispensabile) and be contained in a standing position. The catheter is inserted from the dorsal vulvar commissure (just like the cotton swab for vaginal smear) and its positioning at the end of the vagina is verified through abdominal palpation: the cervix (easily palpable during estrus) is identified and the tip of the catheter must be palpated just caudally. Once all the semen has been flushed from the catheter the hind legs of the bitch are elevated and kept in this position for 5-10 minutes (a procedure which is widely believed to help spermatozoa cross the cervix, although no scientific data have ever been produced).

Frozen semen must be inseminated into the uterus, as thawed spermatozoa are short-lived and cannot move vigorously enough to cross the cervix in numbers adequate to achieve a good conception rate. A Norwegian catheter (made by a steel 2.0 mm catheter with a Teflon sheath) purposedly designed for AI in foxes works well in bitches and has been used at the canine frozen semen bank of the University of Uppsala and at a few other centers throughout the world for the last 12-15 years with good results. One hand identifies and holds the cervix while the other one pushes the Teflon sheath until it reaches the paracervix, after which the steel tip of the catheter is carefully worked through the cervix into the uterus. Disadvantages of this catheter are a difficult learning process (it takes working on canine uterine tracts taken at necropsy and then practicing on a high number of bitches before the technique can be mastered, and not everybody seem to be able or constant enough to learn) and the fact that it cannot be used in large size bitches (because the cervix cannot be palpated with one hand). The French catheter Osiris has an inflatable ballon at the end which blocks the catheter itself within the vagina simulating the coital lock. It is commonly believed that such mechanism improves transit of frozen semen through the canine cervix, although no scientific data are available

The cervix can be passed also with a rigid endoscope. A human cystoscope or cystoureteroscope is best used. A complete set of endoscopy (CO2 insufflator, light source etc.) is necessary, which makes the technique expensive. Alternatively, intrauterine insemination can be performed surgically or laparoscopically. The surgical approach is faster, both are without complications and conceptione rate is in the 60%-70% range.

References
Concannon PW, and Battista – Canine semen freezing and artificial insemination. Current Veterinary Therapy X: Small Animal Practice, 1989, pag 1247;