Use of frozen semen in bitch

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Abstract

Frozen semen was first used successfully in the bitch in 1969. Initially semen was inseminated by vaginal insemination and subsequently, intrauterine techniques were used, achieving higher pregnancy rates than vaginal insemination. For optimal results with freezing and using frozen semen, careful collection, assessment, freezing and handling techniques must be employed. It is important when inseminating frozen semen to consider extender constituents and to make appropriate dilutions with thaw media to reduce glycerol concentrations in the extender to safe levels. However, a difficulty with using frozen semen in dogs is that many extender recipes are proprietary and constituents are unknown. Poor handling of frozen semen straws (through relabelling and holding straws out of liquid nitrogen for > 15 - 20 seconds) detrimentally affect both motility of thawed sperm and membrane integrity and are likely to reduce pregnancy rates

Keywords: Frozen semen, bitch, canine, artificial insemination

Introduction

Frozen semen was first used successfully in the bitch in 1969.[1] Initially semen was inseminated by vaginal insemination,[2] but subsequently, intrauterine techniques were utilised, resulting in higher pregnancy rates than vaginal insemination.[3-5] Initially insemination by laparotomy under general anaesthesia was utilised; however, in 1972, Kell Andersen developed a non-surgical intrauterine insemination technique using the Norwegian catheter.[6] As fresh and fresh chilled semen have higher pregnancy rates than frozen semen, the latter should be reserved for situations wherein use of fresh or chilled semen is impossible or not viable. Since the first successful frozen semen insemination, its use has increased and pregnancy rates have improved. In most parts of the world, frozen semen is used to introduce new genetics from deceased dogs or dogs in remote countries. In some countries, such as Australia and New Zealand, there are strict quarantine laws precluding and inhibiting importation of dogs or semen. Testing of animals to enter such countries is a protracted process, making it uneconomical and not in the best welfare interests of the bitch or dog to move for a short interval for breeding. Chilled semen is ruled out due to testing requirements and so frozen semen is the only viable option.

There are several contributing factors to a successful pregnancy with the use of frozen semen, including fertility of the dog, collection method, freezing method, transport and handling of the semen, fertility of the bitch, timing of the bitch's cycle, and insemination method and the operator responsible for any of these procedures.[7-9] Many of these factors are impossible to control; however, some factors are within the control of the people involved and how these operations are performed can dramatically affect the end result of the insemination.

Semen collection

Ideally only proven fertile males should have their semen frozen. Peak fertility of male dogs is believed to be between 2 and 5 years of age (observed, unpublished data by author), so it is recommended to collect dogs for freezing between these ages. The male should be prevented from urinating in the immediate interval preceding collection, as urination during this time will increase the chance of urospermia during semen collection. Semen should be collected from the male in the presence of an estrous bitch for best results. A comfortable and relaxed environment should be provided for semen collection. Adequate time for the male and female to become acquainted is important for both the dogs to relax and also for the male to become adequately stimulated. Allowing the male to mount the bitch may be beneficial and the male should be minimally restrained with minimal distractions. Manual stimulation of the bulbus glandis through the prepuce and gentle stimulation of the tip of the penis will help stimulate erection and ejaculation. The prepuce should be reflected back over the bulbus glandis by the operator, as obtaining an erection with the bulbus glandis within the prepuce can lead to significant pain which would be expected to reduce semen quality. The dog ejacuates in 3 fractions and the second, semen-rich fraction, should be separated

from the other 2 prostatic fractions. Poorly fractionated samples may be centrifuged and washed before freezing, although it is believed that excessive manipulation of semen may have negative impacts on fertility.

Semen assessment

A difficulty with freezing semen is adequately assessing a sample as suitable to freeze before knowing true parameters. It is not recommended to utilise semen with raw semen values of less than 70% motility, 60% normal morphology, or samples contaminated with blood, pus or urine. In most cases, motility of dog sperm appears to decrease by 20 - 30% from prefreezing values, so a rough calculation should be performed based on total number of sperm, initial motility (to predict motility of thawed sperm) and approximate morphology values to determine if there will be any usable samples after freezing.

Sperm concentration and motility

The sperm-rich fraction is accurately measured to ascertain the volume collected (in general 0.5 -1.5 ml). An aliquot of semen is then taken and diluted to enable accurate assessment of total number of sperm and motility. The author will take 10 µl of the semen rich fraction and add it to 180 µl of thaw media extender (Andropro AI thaw, Minitube, Verona, WI) to obtain a 1:9 dilution. The aim is to end up with a concentration of <50 million sperm/ml to allow for the sperm to be 'free moving' and obtain a more accurate motility value and total count. Dilution rate may be altered depending on the concentration of the original sample. To aid in dilution, approximate sperm concentrations based on colour/density of the gross sample are shown in Table 1.

Table 1. Approximate sperm concentration rates of canine semen samples by gross appearance.

Creamy, grainy semen	750 - 1500 x 10^6 sperm/ml
Full cream milk like semen	400 - 750 x 10^6 sperm/ml
Skim milk like semen	250 - 400 x 10^6 sperm/ml
Translucent semen	< 250 x 10^6 sperm/ml

The author uses a Computer Assisted Semen Analysis (CASA) system (Spermvision, Minitube, Verona, WI) to determine concentration and motility. Other options include a hemocytometer, Makler chamber, Spermacue, iSperm, other CASA systems and the Nucleocounter SP200. For the hemocytometer, Makler chamber and Spermacue, sperm motility assessment is made via visual estimation which may counteract any sperm concentration accuracy. All sperm counting systems have their limitations and must be used appropriately for most accurate results.[10, 11] Total number of sperm in the sample are recorded, as is progressive motility (sperm that swim their body length in 1 second) not total motility (sum of progressive and non-progressively motile sperm).

Sperm morphology

Sperm morphology may be obtained using a wet mount prep or stained slide. A minimum of 100 sperm should be assessed and each sperm categorised into normal and their specific abnormal form. Sperm with multiple abnormalities are classically identified by the abnormality closest to the sperm head. The minimum acceptable standard for canine sperm morphology is 60% normal sperm with no more than 20% of any 1 type of abnormality. Motility of thawed sperm values are proportional to percentage of normal sperm and also initial raw motility values.

Sperm viability

Sperm viability (membrane integrity) is becoming easier to assess in practice using the Hyperosmotic Swelling Test (HOST), live/dead Eosin Nigrosin stains or fluorescent microscopy with viability stains (Propridium Iodide and SYBR Green). Membrane integrity is believed to be related to semen fertility.[12] Semen samples with higher percentage viability should freeze better.

Semen Freezing

Canine semen is frozen in either straws or pellets. Extenders used to freeze semen are usually a 1 or 2 step process, with the latter being more common. Sperm frozen at 200 x 10^6/ml is likely to

yield better results for thawed samples than other concentrations.[13] Semen extenders contain a sugar source (usually fructose), a buffer to control pH, antibiotics (to protect the medium from growth, not prevent infection in the bitch), a membrane stabiliser (egg yolk or skim milk) and a cryoprotectant (glycerol). Glycerol is one of the most important constituents to consider with using frozen semen effectively. To protect semen from freeze-thaw damage, extender needs to contain a minimum of 4 % glycerol.[14] However, once thawed for insemination, if the glycerol concentration is > 2.5%, longevity of thawed sperm is reduced.[13] There are several commercial extenders with differing freeze protocols that need to be followed. Unfortunately, most are proprietary recipes and concentration of glycerol is not disclosed. Some of the more common canine semen freezing extenders and glycerol concentrations of those that are known are shown in Table 2.

Table 2. Semen extenders and glycerol concentration.

Extender	Glycerol (%, w/v)
CLONE	Unknown
CaniRep (Uppsala)	7.5
CaniPro (Minitube, Verona, WI)	4
Synbiotics (Zoetis)	Unknown
Synthetic (IMV)	8
Triladyl	4
Camelot	Unknown
ICSB	Unknown

Semen freezing extenders which are frozen containing egg yolk before distribution appear to result in more sperm agglutination than those which utilise fresh egg yolk addition at the time of freezing (Observed unpublished data by author). Each semen freeze extender has a recipe that must be followed to create extended semen ready for freezing.

Whilst semen and extenders are equilibrating, straws or vials need to be prepared and permanently labelled for later identification. It is recommended to label straws or vials with the dog's name, breed, microchip number and date in long format (e.g. 15th January 2019).

Semen freezing in pellets

Dry ice is utilised to freeze semen. Small holes are made in the surface of the dry ice. The extended semen sample is distributed 1 drop at a time into each well using a glass Pasteur pipette, whilst continually mixing the extended semen to ensure sperm don't settle. Each drop will snap freeze to form a pellet, which are then transferred off the dry ice into liquid nitrogen. One pellet is thawed in thaw media and motility assessed. Once this is done, the number of pellets required for an insemination can be calculated: [total number of sperm x % morphologically normal sperm x motility after thawing (%)]/Number pellets. Pellets are then distributed into separate vials, with each vial being intended for 1 insemination.

Semen freezing in straws

Semen and extender mix are refrigerated to 4°C. Labelled straws are also equilibrated to 4°C. Each straw is filled with 0.5 ml of extended semen and the fluid is brought up to the wick of the straw to proceed to the cement. The other end of the straw is sealed and then the straw is given a short flick to move the air bubble from the sealed end to the centre of the straw (this prevents straws from exploding on thawing).

Thawing semen for insemination

Pellets

Pellets are emptied into a whirl pack bag containing either 1 ml of saline or thaw media in a 37°C water bath. Pellets are massaged through the bag to encourage thawing. The author uses a step thaw technique, wherein pellets are added to 1 ml of warmed thaw media (AI thaw, Minitube, Verona, WI) and then an additional 2 ml of thaw media is slowly added. This technique slowly changes osmolarity of the semen extender and improves motility of thawed sperm.[13]

Straws

Straws may be thawed at 37°C for 1 minute or 70°C for 8 seconds, with the latter resulting in greater longevity of thawed samples.[15] Thawed semen is then emptied into a whirl pack bag containing thaw media at 37°C. The amount of thaw media to use is recommended by the extender manufacturer and should come as instructions with semen. However, as semen often arrives with no paperwork, the amount of thaw media used for various extenders by the author is shown in Table 3.

Table 3. Volume of thaw media (AndroPro AI, Minitube, Verona, WI) used by author when inseminating frozen semen.

Extender	Volume AndroPro AI (ml) per straw
CLONE	1.5
CaniRep (Uppsala)	1.5
CaniPro (Minitube, Verona, WI)	0.5
Synbiotics (Zoetis)	1-1.5
Synthetic (IMV)	1.5
Triladyl	0.5
Camelot	3 (1 then 2 ml step thaw)
ICSB	3 (1 then 2 ml step thaw)

Insemination of frozen semen

It is universally accepted that for best results, frozen semen should be inseminated intrauterine in bitches.[3,8,9,16,17] Whilst TCI is more successful than surgical intrauterine insemination if performed appropriately,[8] the method of insemination should be 1 with which the operator is most confident. It is accepted that the lifespan of frozen semen is indefinite while frozen. However, as sperm are not dead, there will still be consumption of energy sources from the frozen semen extender and accumulation of metabolic waste products over time. Therefore, for older frozen semen to be successfully used, more semen than originally planned may be needed.

It is well published that for best pregnancy results a minimum of 150 million live, morphologically normal sperm should be inseminated on 1 occasion if the estrous cycle is timed well.[3,18-21] However, if the estrous cycle is timed poorly, then 2 inseminations 24 - 48 hours apart of the aforementioned number of motile sperm should be inseminated.[20,21] If estrous cycle timing is well done, then the only benefit to an additional insemination is an average of 1 additional puppy per litter.[20,21] The universally accepted value is to inseminate 100 million live, morphologically normal sperm; however, there are limited published data to support this.[8] Although some operators advocate the use of differing numbers of sperm for different-sized bitches, to the author's knowledge, there are no good published data to support this.

To ensure enough sperm are inseminated, the recommended number of straws (or vials) should be thawed when the insemination is ready to occur. The author uses CASA (Spermvision, Minitube, Verona, WI) to assess semen, due to its reliability and speed. For this, 3 µl of thawed semen is placed in a Leja chamber and analysed for concentration and motility. If the number of motile sperm is <100 million, then more semen is thawed (if available) until >100 million motile sperm are attained. The final volume is then inseminated via TCI. If surgical insemination is used, the sample would have to be centrifuged to a more usable volume and then inseminated.

An alternative to CASA is to quickly count number of sperm in the sample using a Makler chamber. For this, 20 µl of thawed semen is added to 5 µl of 5% formalin saline and 10 µl of the immobilised solution placed in the Makler chamber for analysis. Motility is guesstimated from the original sample and then number of motile sperm in the thawed sample calculated.

Timing the estrous cycle for insemination of frozen semen

Timing of the estrous cycle to determine the best time for insemination of frozen semen is best undertaken using a combination of vaginal cytology, progesterone assay and vaginoscopy. Althogh LH assays may be beneficial, compared to the other 3 techniques, their use requires more frequent visits and blood sampling to not miss the LH surge.[22,23] For best results, frozen semen is inseminated after ovulation, when maximal crenulation of the vagina is evident on vaginoscopy, before the bitch enters diestrus.[9]

Handling effects of frozen semen on motility and likely fertility

Frozen semen straws or vials are recommended to be stored in liquid nitrogen or liquid nitrogen vapour for minimal disturbance to sperm quality.

Some technicians advocate relabelling straws that have been incorrectly identified or inappropriately labelled, which will restrict the import/export of frozen semen. Special FreezerBonz II® (Brady Labxpert, Mexico) labels are used as they will stick to frozen straws. To determine if relabelling of semen straws affects sperm motility and quality, 9 straws of frozen semen were individually cut in half within a bath of liquid nitrogen. One of the halves of each straw was relabelled by quickly removing it from the liquid nitrogen, wiping the surface with a tissue to remove ice and then applying the FreezerBondz II® label and then this half straw was immediately returned to the liquid nitrogen bath. The process of relabelling took <20 seconds and contents remained visually frozen. Each half straw was then individually thawed in 0.5 ml Andropro AI (Minitube, Verona, WI) in a water bath at 37°C for 1 minute. Motility of each sample was assessed using CASA (Spermvision, Minitube, Verona, WI). Viability (membrane integrity) was assess using CYBR14/PI staining using fluorescent microscopy. Results are shown in Table 4.

Based on paired sample Student's t test, there were differences ($p < 0.05$) between relabelled and non-relabelled straws for both motility and viability. This would indicate that this procedure should not be performed, as it is likely to reduce fertility. Furthermore, removal of straws/vials from liquid nitrogen should be for very short intervals to avoid damaging semen.

Table 4. Motility (%) and viability (%) results of each relabelled (label) and non-relabelled (no label) half straw.

	Motility 1 minute		Motility 2 minutes		Motility 10 minutes		Viability	
Straw number	No Label	Label	No label	Label	No label	Label	No Label	Label
1	47	33	43	34	48	42		
2	36	31	43	42	45	37		
3	36	10	22	11	33	9	25.7	4
4	39	26	40	21	31	20	31.4	10.8
5	49	29	45	27	45	29	44.1	20.7
6	48	46	47	46	53	43	41.9	25
7	50	40	50	38	48	41	54	14.9
8	52	33	57	34	52	36	50	22.1

Conclusion

Whilst a thorough understanding of the techniques of freezing and thawing dog semen is important, it is equally important to have an understanding of extender constituents to ensure semen is being handled adequately to maximise pregnancy rates. Frozen semen handling is equally important, as relabelling or holding frozen straws or pellets out of liquid nitrogen for even short intervals will decrease sperm quality.

Conflict of interest

None to declare.

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